Docket No.: 04280/1201643-US2
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Letters Patent of:
Nils Lonberg et al.

Patent No.: 7,084,260

Issued: August 1, 2006

For: TRANSGENIC NON-HUMAN ANIMALS FOR PRODUCING HETEROLOGOUS ANTIBODIES

REQUEST FOR CERTIFICATE OF CORRECTION
PURSUANT TO 37 CFR 1.323 AND 1.322

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Upon reviewing the above-identified patent, Patentee noted typographical errors which should be corrected. A listing of the errors to be corrected is attached.

The typographical errors marked with an "A" on the attached list are found in the application as filed by applicant. Please charge our Credit Card in the amount of $100.00 covering the fee set forth in 37 CFR 1.20(a).

The typographical errors marked with an "P" on the attached list are not in the application as filed by applicant. Also given on the attached list are the documents from the file history of the subject patent where the correct data can be found.

The errors now sought to be corrected are inadvertent typographical errors the correction of which does not involve new matter or require reexamination.

Transmitted herewith is a proposed Certificate of Correction effecting such corrections. Patentee respectfully solicits the granting of the requested Certificate of Correction.

The Commissioner is authorized to charge any deficiency of up to $300.00 or credit any excess in this fee to Deposit Account No. 04-0100.

Dated: February 26, 2007

Respectfully submitted,

By _____

Flynn Barrison
Registration No.: 53,970
DARBY & DARBY P.C.
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(212) 527-7701 (Fax)
Attorneys/Agents For Applicant

PTO/SB/44 (04-05)
Approved for use through 04/30/2007. OMB 0651-0033
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.	:	7,084,260
APPLICATION NO.	:	08/728,463
ISSUE DATE	:	August 1, 2006
INVENTOR(S)	:	Nils Lonberg et al.

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

First Page Col. 1 (Other Publications); Line 1; Delete "et al" and insert - - et al., - -, therefor.

First Page Col. 1 (Other Publications); Line 1; Delete "Immunology" and insert - - Immunobiology - -, therefor.

First Page Col. 1 (Other Publications); Line 5; Delete "et. al.," and insert - - et al., - -, therefor.

First Page Col. 1 (Other Publications); Line 8; Delete "et. al.," and insert - - et al., - -, therefor.

First Page Col. 2 (Other Publications); Line 30; Delete "(19000." and insert - - (1990). - -, therefor.

Page 2 Col. 1 (Other Publications); Line 4; Delete "an" and insert - - and - -, therefor.

Page 2 Col. 1 (Other Publications); Line 19; Delete "89329-8935" and insert - - 8932-8935 - -, therefor.

Page 2 Col. 1 (Other Publications); Line 21; Delete "introducted" and insert - - introduced - -, therefor.

Page 2 Col. 1 (Other Publications); Line 29; Delete "K" and insert - - κ - -, therefor.

Page 2 Col. 1 (Other Publications); Line 39; Delete "et.al.," and insert - - et al., - -, therefor.

Page 2 Col. 2 (Other Publications); Line 2; Delete "genesJ." and insert - - genes J. - -, therefor.

Page 2 Col. 2 (Other Publications); Line 34; Delete "γ" and insert - - k - -, therefor.

Page 2 Col. 2 (Other Publications); Line 38; Delete "chin" and insert - - chain - -, therefor.

MAILING ADDRESS OF SENDER (Please do not use customer number below):
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New York, New York 10150-5257

Page 2 Col. 2 (Other Publications); Line 49; Delete "et. al.," and insert - - et al., - -, therefor.

Page 2 Col. 2 (Other Publications); Line 54; Delete "tandes" and insert - - tandem - -, therefor.

Page 2 Col. 2 (Other Publications); Line 59; Delete "et. al.," and insert - - et al., - -, therefor.

Page 3 Col. 1 (Other Publications); Line 3; Delete "Acad" and insert - - Acad. - -, therefor.

Page 3 Col. 1 (Other Publications); Line 13; Delete "135::620-626" and insert - - 135:620-626 - -, therefor.

Page 3 Col. 1 (Other Publications); Line 18; Delete "immnology" and insert - - immunology - -, therefor.

Page 3 Col. 1 (Other Publications); Line 39; Delete "unrearrange" and insert - - unrearranged - -, therefor.

Page 3 Col. 1 (Other Publications); Line 47; Delete "nature" and insert - - Nature - -, therefor.

Page 3 Col. 1 (Other Publications); Line 60; Delete "86:5567-5572" and insert - - 86:5567-5571 - -, therefor.

Page 3 Col. 2 (Other Publications); Line 4; Delete "immunologlobulin" and insert - - immunoglobulin - -, therefor.

Page 3 Col. 2 (Other Publications); Line 5; Delete "developemtn" and insert - - development - -, therefor.

Page 3 Col. 2 (Other Publications); Line 12; Delete "nature" and insert - - Nature - -, therefor.

Page 3 Col. 2 (Other Publications); Line 13; Delete "Morrision," and insert - - Morrison, - -, therefor.

Page 3 Col. 2 (Other Publications); Line 15; Delete "Petterson" and insert - - Pettersson - -, therefor.

Page 3 Col. 2 (Other Publications); Line 33; Delete "Vlassov" and insert - - Vlasov - -, therefor.

Page 3 Col. 2 (Other Publications); Line 43; Delete "Chan" and insert - - Chen - -, therefor.

Page 3 Col. 2 (Other Publications); Line 47; Delete "Ami-CD4" and insert - - Anti-CD4 - -, therefor.

Page 3 Col. 2 (Other Publications); Line 57; Delete "V_x" and insert - - V_H - -, therefor.

Page 3 Col. 2 (Other Publications); Line 58; Delete "V_μ" and insert - - V_H - -, therefor.

Page 3 Col. 2 (Other Publications); Line 59; Delete "loops." and insert - - Loops. - -, therefor.

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Sheet 22 of 99 (FIG.21A); Line 1; Delete " HEAVY " and insert - - HEAVY - -, therefor.

Column 2; Line 60; Delete "e.g." and insert - - e.g., - -, therefor.

Column 5; Line 57; Delete "e.g." and insert - - e.g., - -, therefor.

Column 6; Line 40; Delete "engognous" and insert - - endogenous - -, therefor.

Column 6; Line 65; Delete "transgehne," and insert - - transgene, - -, therefor.

Column 7; Line 67; Delete "gene," and insert - - gene. - -, therefor.

Column 8; Line 1; Delete "locus," and insert - - locus. - -, therefor.

Column 8; Line 2; Delete "locus," and insert - - locus. - -, therefor.

Column 8; Line 3; Delete "locus," and insert - - locus. - -, therefor.

Column 10; Line 58; Delete "calorimetric" and insert - - colorimetric - -, therefor.

Colum 13; Line 13; After "4437;" insert - -) - -.

Column 13; Line 15; Delete "4437." and insert - - 4437). - -, therefor.

Column 14; Line 60-61 (Approx.); Delete "Table 17. Rate.....human CD4" and insert the same on line 62 as a new paragraph.

Column 15; Line 39; Delete "chainsspecifically" and insert - - chains specifically - -, therefor.

Column 16; Line 56; Delete "immunogloblins" and insert - - immunoglobulins - -, therefor.

Column 20; Line 37; Delete "e.g." and insert - - e.g., - -, therefor.

Column 21; Line 20; After "subclasses" insert - - . - -.

Column 21; Line 21; Delete "Σ" and insert - - ϵ - -, therefor.

Column 25; Line 31; Delete "Non-human" and insert - - Non-Human - -, therefor.

Column 25; Line 52; Delete "e.g." and insert - - e.g., - -, therefor.

Column 26; Line 53; Delete "e.g." and insert - - e.g., - -, therefor.

Column 26; Line 58; Delete "e.g." and insert - - e.g., - -, therefor.

Column 27; Line 51; Delete "e.g." and insert - - e.g., - -, therefor.

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Column 35; Line 8; Delete "μ," and insert - - p, - -, therefor.

Column 35; Line 57; Delete "cH" and insert - - C_H - -, therefor.

Column 38; Line 24; Delete "CL" and insert - - C_L - -, therefor.

Column 40; Line 43-52; Delete "such depletion can be.....antibody, and the like." and insert the same on line 42 as a continuation of paragraph.

Column 40; Line 62-64; Delete "thus, a suppression.....in transgenic mice." and insert the same on line 61 as a continuation of paragraph.

Column 42; Line 13 (Approx.); Delete "Trans-switching" and insert - - Trans-Switching - -, therefor.

Column 42; Line 35; Delete "adavantages" and insert - - advantages - -, therefor.

Column 44; Line 53; Delete "u)" and insert - - μ) - -, therefor.

Column 45; Line 53; Delete "e.g." and insert - - e.g., - -, therefor.

Column 46; Line 44; Delete "antibodys" and insert - - antibodies - - therefor.

Column 47; Line 8; Delete "monocloanl" and insert - - monoclonal - -, therefor.

Column 47; Line 14; Delete "(e.g." and insert - - (e.g., - -, therefor.

Column 49; Line 3; Delete "e.g." and insert - - e.g., - -, therefor.

Column 49; Line 40; Delete "CDRS," and insert - - CDRs, - -, therefor.

Column 49; Line 55; Delete "$5x10^{10}M^{-1}M^{-1}$" and insert - - $5x10^{10}M^{-1}$ - -, therefor.

Column 50; Line 4; Delete "sequene" and insert - - sequence - -, therefor.

Column 50; Line 11; Delete "sequene" and insert - - sequence - -, therefor.

Column 50; Line 38; Delete "sequene" and insert - - sequence - -, therefor.

Column 50; Line 45; Delete "sequene" and insert - - sequence - -, therefor.

Column 50; Line 65; Delete "sequene" and insert - - sequence - -, therefor.

Column 51; Line 5; Delete "sequene" and insert - - sequence - -, therefor.

Column 51; Line 18; Delete "transmemebrane" and insert - - transmembrane - -, therefor.

Column 52; Line 22; After "transgene" insert - - . - -.

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Column 52; Line 46; Delete "(i.e," and insert - - (i.e., - -, therefor.

Column 54; Line 33; Delete "e.g." and insert - - e.g., - -, therefor.

Column 55; Line 21; Delete "full-lenght" and insert - - full-length - -, therefor.

Column 55; Line 31; Delete "practicioner," and insert - - practitioner, - -, therefor.

Column 58; Line 61; Delete "the;agarose" and insert - - the agarose - -, therefor.

Column 59; Line 58; Delete "Mini-locus" and insert - - Mini-Locus - -, therefor.

Column 62; Line 59; Delete "fragment." and insert - - fragment - -, therefor.

Column 71; Line 25; Delete "(FIG. 2c)." and insert - - (FIG. 20C). - -, therefor.

Column 71; Line 54; Delete "p." and insert - - pp. - -, therefor.

Column 72; Line 54; Delete "p." and insert - - pp. - -, therefor.

Column 74; Line 8; Delete "poli," and insert - - poli, - -, therefor.

Column 74; Line 28; Delete "PGMT-TK" and insert - - pGMT-TK - -, therefor.

Column 75; Line 49; Delete "p." and insert - - pp. - -, therefor.

Column 76; Line 67; After "below" delete "pGPla".

Column 79; Line 49; Delete "deleteon" and insert - - deletion - -, therefor.

Column 80; Line 52; Delete "ou" and insert - - $\sigma\mu$ - -, therefor.

Column 101; Line 57; Delete "immmunoglbulins" and insert - - immunoglobulins - -, therefor.

Column 102; Line 46; Delete "pNNO3." and insert - - pNN03. - -, therefor.

Column 105; Line 4; Delete "(Cyrstal" and insert - - (Crystal - -, therefor.

Column 107; Line 27; Delete "e.g." and insert - - e.g., - -, therefor.

Column 108; Line 8; Delete "1-50," and insert - - 10-50, - -, therefor.

Column 108; Line 37; Delete "West" and insert - - Westr - -, therefor.

Column 109; Line 32; Delete "Of" and insert - - of - -, therefor.

Column 109; Line 63; After "mouse μ" insert - - and - -.

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Column 110; Line 23; Delete "IgX" and insert - - Igλ - -, therefor.

Column 110; Line 65; Delete "intergration" and insert - - integration - -, therefor.

Column 111; Line 2; Delete "intergration" and insert - - integration - -, therefor.

Column 111; Line 25-26; Delete "immmunoglobulin" and insert - - immunoglobulin - -, therefor.

Column 111; Line 37; Delete "immmunoglobulin" and insert - - immunoglobulin - -, therefor.

Column 113; Line 56; Delete "(i.e," and insert - - (i.e., - -, therefor.

Column 113; Line 59; Delete "(i.e," and insert - - (i.e., - -, therefor.

Column 115; Line 57; Delete "reconstitutued" and insert - - reconstituted - -, therefor.

Column 117; Line 8; Delete "phosphotransferse" and insert - - phosphotransferase - -, therefor.

Column 120; Line 53; Delete "J_H3," and insert - - JH3, - -, therefor.

Column 120; Line 54; Delete "Eu, Su," and insert - - Eμ, Sμ, - -, therefor.

Column 123; Line 29; Delete "IgX" and insert - - Igλ - -, therefor.

Column 124; Line 45; Delete "Trans-switching" and insert - - Trans-Switching - -, therefor.

Column 126; Line 42; Delete "seqeunce" and insert - - sequence - -, therefor.

Column 128; Line 7; Delete "31" and insert - - 3' - -, therefor.

Column 128; Line 10; Delete "31" and insert - - 3' - -, therefor.

Column 128; Line 12; Delete "31" and insert - - 3' - -, therefor.

Column 129; Line 2; Delete "Igu" and insert - - Igμ - -, therefor.

Column 129; Line 3; Delete "Igu" and insert - - Igμ - -, therefor.

Column 130; Line 62; Delete "IgEK" and insert - - IgEκ - -, therefor.

Column 130; Line 64; Delete "IgEK" and insert - - IgEκ - -, therefor.

Column 130; Line 67; Delete "λCoated Plates" and insert - - λ Coated Plates. - -, therefor.

Column 131; Line 9; After "IgG" insert - - . - -.

Column 131; Line 54; Delete "Anti-human" and insert - - Anti-Human - -, therefor.

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Column 132; Line 60; Delete "u" and insert - - μ - -, therefor.

Column 132; Line 64; Delete "u" and insert - - μ - -, therefor.

Column 133; Line 51; Delete "inununoglobuhn" and insert - - immunoglobulin - -, therefor.

Column 134; Line 58; Delete "V_H" and insert - - VH - -, therefor.

Column 135; Line 3; Delete "K" and insert - - κ - -, therefor.

Column 140; Line 34; Delete "15 ng" and insert - - 15 ng/ml, - -, therefor.

Column 144; Line 28; Delete "$B202^{bright}$" and insert - - $B220^{bright}$ - -, therefor.

Column 145; Line 27-46; Delete "Wells.....(ABT). For the.....(Jackson). Murine.....205). Appropriate antibodies.....followed." and insert the same on line 26 as a continuation of paragraph.

Column 146; Line 30; Delete "Eor" and insert - - For - -, therefor.

Column 146; Line 57; Delete "absorbtivity" and insert - - absorptivity - -, therefor.

Column 148; Line 2; Delete "calorimetric" and insert - - colorimetric - -, therefor.

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Issued Patent Proofing Form
Note: **P** = PTO Error **A** = Applicant Error

File#: **04280/1201643-US2**

US Serial No.: **08/728,463** US Patent No.: **US 7,084,260 B1** Issue Dt.: **Aug. 1, 2006**

Title: **HIGH AFFINITY HUMAN ANTIBODIES AND HUMAN ANTIBODIES AGAINST HUMAN ANTIGENS**

Sr. No.	P/A	Original		Issued Patent		Description Of Error
		Page	Line	Column	Line	
1	P	Page 1 of 1 List of references cited by examiner (12/08/1998)	Entry 1 Line 1 (Non-Patent Documents)	First Page Col. 1 (Other Publications)	1	Delete "et al" and insert - - et al., - -, therefor.
2	P	Page 1 of 1 List of references cited by examiner (12/08/1998)	Entry 1 Line 1 (Non-Patent Documents)	First Page Col. 1 (Other Publications)	1	Delete "Immunology" and insert - - Immunobiology - -, therefor.
3	A	Page 1 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 2 Line 1 (Other Art)	First Page Col. 1 (Other Publications)	5	Delete "et. al.," and insert - - et al., - -, therefor.
4	A	Page 1 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 3 Line 1 (Other Art)	First Page Col. 1 (Other Publications)	8	Delete "et. al.," and insert - - et al., - -, therefor.
5	P	Page 3 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 2 Line 2 (Other Art)	First Page Col. 2 (Other Publications)	30	Delete "(19000." and insert - - (1990). - -, therefor.
6	P	Page 3 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 10 Line 1 (Other Art)	Page 2 Col. 1 (Other Publications)	4	Delete "an" and insert - - and - -, therefor.
7	P	Page 4 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 4 Line 3 (Other Art)	Page 2 Col. 1 (Other Publications)	19	Delete "89329-8935" and insert - - 8932-8935 - -, therefor.
8	P	Page 4 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 5 Line 2 (Other Art)	Page 2 Col. 1 (Other Publications)	21	Delete "introducted" and insert - - introduced - -, therefor.

9	P	Page 4 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 12 Line 1 (Other Art)	Page 2 Col. 1 (Other Publications)	29	Delete "K" and insert - - κ - -, therefor.
10	A	Page 5 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 2 Line 1 (Other Art)	Page 2 Col. 1 (Other Publications)	39	Delete "et.al.," and insert - - et al., - -, therefor.
11	A	Page 6 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 2 Line 2 (Other Art)	Page 2 Col. 2 (Other Publications)	2	Delete "genesJ." and insert - - genes J. - -, therefor.
12	P	Page 7 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 2 Line 2 (Other Art)	Page 2 Col. 2 (Other Publications)	34	Delete "γ" and insert - - κ - -, therefor.
13	P	Page 7 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 3 Line 3 (Other Art)	Page 2 Col. 2 (Other Publications)	38	Delete "chin" and insert - - chain - -, therefor.
14	A	Page 7 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 7 Line 1 (Other Art)	Page 2 Col. 2 (Other Publications)	49	Delete "et. al.," and insert - - et al., - -, therefor.
15	P	Page 7 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 8 Line 2 (Other Art)	Page 2 Col. 2 (Other Publications)	54	Delete "tandes" and insert - - tandem - -, therefor.
16	A	Page 8 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 1 Line 1 (Other Art)	Page 2 Col. 2 (Other Publications)	59	Delete "et. al.," and insert - - et al., - -, therefor.
17	A	Page 8 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 4 Line 3 (Other Art)	Page 3 Col. 1 (Other Publications)	3	Delete "Acad" and insert - - Acad. - -, therefor.

18	P	Page 8 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 7 Line 3 (Other Art)	Page 3 Col. 1 (Other Publications)	13	Delete "135::620-626" and insert - - 135:620-626 - -, therefor.
19	P	Page 8 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 9 Line 2 (Other Art)	Page 3 Col. 1 (Other Publications)	18	Delete "immnology" and insert - - immunology - -, therefor.
20	P	Page 9 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 7 Line 2 (Other Art)	Page 3 Col. 1 (Other Publications)	39	Delete "unrearrange" and insert - - unrearranged - -, therefor.
21	P	Page 9 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 9 Line 3 (Other Art)	Page 3 Col. 1 (Other Publications)	47	Delete "nature" and insert - - Nature - -, therefor.
22	P	Page 10 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 3 Line 3 (Other Art)	Page 3 Col. 1 (Other Publications)	60	Delete "86:5567-5572" and insert - - 86:5567-5571 - -, therefor.
23	P	Page 10 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 6 Line 2 (Other Art)	Page 3 Col. 2 (Other Publications)	4	Delete "immunologlobulin" and insert - - immunoglobulin - -, therefor.
24	P	Page 10 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 6 Line 2 (Other Art)	Page 3 Col. 2 (Other Publications)	5	Delete "developemtn" and insert - - development - -, therefor.
25	P	Page 10 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 8 Line 3 (Other Art)	Page 3 Col. 2 (Other Publications)	12	Delete "nature" and insert - - Nature - -, therefor.
26	P	Page 10 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 9 Line 1 (Other Art)	Page 3 Col. 2 (Other Publications)	13	Delete "Morrision," and insert - - Morrison, - -, therefor.

27	P	Page 10 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 10 Line 1 (Other Art)	Page 3 Col. 2 (Other Publications)	15	Delete "Petterson" and insert - - Pettersson - -, therefor.
28	P	Page 11 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 6 Line 1 (Other Art)	Page 3 Col. 2 (Other Publications)	33	Delete "Vlassov" and insert - - Vlasov - -, therefor.
29	P	Page 12 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 1 Line 1 (Other Art)	Page 3 Col. 2 (Other Publications)	43	Delete "Chan" and insert - - Chen - -, therefor.
30	P	Page 12 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 2 Line 2 (Other Art)	Page 3 Col. 2 (Other Publications)	47	Delete "Ami-CD4" and insert - - Anti-CD4 - -, therefor.
31	P	Page 12 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 5 Line 2 (Other Art)	Page 3 Col. 2 (Other Publications)	57	Delete "V_κ" and insert - - V_H - -, therefor.
32	P	Page 12 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 5 Line 2 (Other Art)	Page 3 Col. 2 (Other Publications)	58	Delete "V_μ" and insert - - V_H - -, therefor.
33	P	Page 12 of 12 List of References cited by applicant and considered by examiner (12/30/2005)	Entry 5 Line 3 (Other Art)	Page 3 Col. 2 (Other Publications)	59	Delete "loops." and insert - - Loops. - - , therefor.
34	A	Sheet 22 of 99 Drawings (07/08/2002)	1 (FIG.21A)	Sheet 22 of 99 (FIG.21A)	1	Delete " HEAVY " and insert - - HEAVY - -, therefor.
35	A	Page 5 Specification (10/10/1996)	31	2	60	Delete "e.g." and insert - - e.g., - -, therefor.
36	A	Page 10 Specification (10/10/1996)	32	5	57	Delete "e.g." and insert - - e.g., - -, therefor.
37	A	Page 12 Specification (10/10/1996)	5	6	40	Delete "engognous" and insert - - endogenous - -, therefor.

38	A	Page 12 Specification (10/10/1996)	29	6	65	Delete "transgehne," and insert - - transgene, - -, therefor.
39	A	Page 14 Specification (10/10/1996)	19	7	67	Delete "gene," and insert - - gene. - -, therefor.
40	A	Page 14 Specification (10/10/1996)	20	8	1	Delete "locus," and insert - - locus. - -, therefor.
41	A	Page 14 Specification (10/10/1996)	21	8	2	Delete "locus," and insert - - locus. - -, therefor.
42	A	Page 14 Specification (10/10/1996)	22	8	3	Delete "locus," and insert - - locus. - -, therefor.
43	P	Page 19 Specification (10/10/1996)	12	10	58	Delete "calorimetric" and insert - - colorimetric - -, therefor.
44	A	Page 23 Specification (10/10/1996)	9	13	13	After "4437;" insert - -) - -.
45	A	Page 23 Specification (10/10/1996)	11	13	15	Delete "4437." and insert - - 4437). - -, therefor.
46	P	Page 26 Specification (10/10/1996)	3-4	14	60-61 (Approx.)	Delete "Table 17. Rate.....human CD4" and insert the same on line 62 as a new paragraph.
47	A	Page 27 Specification (10/10/1996)	9	15	39	Delete "chainsspecifically" and insert - - chains specifically - -, therefor.
48	A	Page 29 Specification (10/10/1996)	11	16	56	Delete "immunogloblins" and insert - - immunoglobulins - -, therefor.
49	A	Page 35 Specification (10/10/1996)	30	20	37	Delete "e.g." and insert - - e.g., - -, therefor.
50	A	Page 37 Specification (10/10/1996)	2	21	20	After "subclasses" insert - - . - -.
51	P	Page 37 Specification (10/10/1996)	3	21	21	Delete "Σ" and insert - - ϵ - -, therefor.
52	P	Page 44 Specification (10/10/1996)	4	25	31	Delete "Non-human" and insert - - Non-Human - -, therefor.
53	A	Page 44 Specification (10/10/1996)	24	25	52	Delete "e.g." and insert - - e.g., - -, therefor.
54	A	Page 46 Specification (10/10/1996)	15	26	53	Delete "e.g." and insert - - e.g., - -, therefor.
55	A	Page 46 Specification (10/10/1996)	20	26	58	Delete "e.g." and insert - - e.g., - -, therefor.

56	A	Page 48 Specification (10/10/1996)	4	27	51	Delete "e.g." and insert - - e.g., - -, therefor.
57	P	Page 61 Specification (10/10/1996)	2	35	8	Delete "μ," and insert - - p, - -, therefor.
58	A	Page 62 Specification (10/10/1996)	15	35	57	Delete "cH" and insert - - C_H - -, therefor.
59	P	Page 4 Specification (08/31/2005)	23	38	24	Delete "CL" and insert - - C_L - -, therefor.
60	P	Page 70 Specification (10/10/1996)	17-26	40	43-52	Delete "such depletion can be.....antibody, and the like." and insert the same on line 42 as a continuation of paragraph.
61	P	Page 70 Specification (10/10/1996)	35-37	40	62-64	Delete "thus, a suppression.....in transgenic mice." and insert the same on line 61 as a continuation of paragraph.
62	P	Page 73 Specification (10/10/1996)	6	42	13 (Approx.)	Delete "Trans-switching" and insert - - Trans-Switching - -, therefor.
63	A	Page 73 Specification (10/10/1996)	27	42	35	Delete "adavantages" and insert - - advantages - -, therefor.
64	P	Page 77 Specification (10/10/1996)	22	44	53	Delete "u)" and insert - - μ) - -, therefor.
65	A	Page 79 Specification (10/10/1996)	10	45	53	Delete "e.g." and insert - - e.g., - -, therefor.
66	A	Page 80 Specification (10/10/1996)	29	46	44	Delete "antibodys" and insert - - antibodies - - therefor.
67	A	Page 81 Specification (10/10/1996)	22	47	8	Delete "monocloanl" and insert - - monoclonal - -, therefor.
68	A	Page 81 Specification (10/10/1996)	27	47	14	Delete "(e.g." and insert - - (e.g., - -, therefor.
69	A	Page 84 Specification (10/10/1996)	30	49	3	Delete "e.g." and insert - - e.g., - -, therefor.
70	A	Page 85 Specification (10/10/1996)	27	49	40	Delete "CDRS," and insert - - CDRs, - -, therefor.
71	P	Page 86 Specification (10/10/1996)	3-4	49	55	Delete "$5x10^{10}M^{-1}M^{-1}$" and insert - - $5x10^{10}M^{-1}$ - -, therefor.
72	A	Page 86 Specification (10/10/1996)	18	50	4	Delete "sequene" and insert - - sequence - -, therefor.

73	A	Page 86 Specification (10/10/1996)	25	50	11	Delete "sequene" and insert - - sequence - -, therefor.
74	A	Page 87 Specification (10/10/1996)	12	50	38	Delete "sequene" and insert - - sequence - -, therefor.
75	A	Page 87 Specification (10/10/1996)	19	50	45	Delete "sequene" and insert - - sequence - -, therefor.
76	A	Page 87 Specification (10/10/1996)	38	50	65	Delete "sequene" and insert - - sequence - -, therefor.
77	A	Page 88 Specification (10/10/1996)	7	51	5	Delete "sequene" and insert - - sequence - -, therefor.
78	A	Page 88 Specification (10/10/1996)	19	51	18	Delete "transmemebrane" and insert - - transmembrane - -, therefor.
79	A	Page 90 Specification (10/10/1996)	12	52	22	After "transgene" insert - - . - -.
80	A	Page 90 Specification (10/10/1996)	36	52	46	Delete "(i.e," and insert - - (i.e., - -, therefor.
81	A	Page 93 Specification (10/10/1996)	38	54	33	Delete "e.g." and insert - - e.g., - -, therefor.
82	A	Page 95 Specification (10/10/1996)	18	55	21	Delete "full-lenght" and insert - - full-length - -, therefor.
83	A	Page 95 Specification (10/10/1996)	27	55	31	Delete "practicioner," and insert - - practitioner, - -, therefor.
84	P	Page 101 Specification (10/10/1996)	22	58	61	Delete "the;agarose" and insert - - the agarose - -, therefor.
85	P	Page 103 Specification (10/10/1996)	4	59	58	Delete "Mini-locus" and insert - - Mini-Locus - -, therefor.
86	P	Page 108 Specification (10/10/1996)	20	62	59	Delete "fragment." and insert - - fragment - -, therefor.
87	P	Page 123 Specification (10/10/1996)	20	71	25	Delete "(FIG. 2c)." and insert - - (FIG. 20C). - -, therefor.
88	A	Page 124 Specification (10/10/1996)	11	71	54	Delete "p." and insert - - pp. - -, therefor.
89	A	Page 126 Specification (10/10/1996)	2	72	54	Delete "p." and insert - - pp. - -, therefor.
90	P	Page 128 Specification (10/10/1996)	11	74	8	Delete "poli," and insert - - poll, - -, therefor.

90	P	Page 128 Specification (10/10/1996)	30	74	28	Delete "PGMT-TK" and insert - - pGMT-TK - -, therefor.
92	A	Page 130 Specification (10/10/1996)	24	75	49	Delete "p." and insert - - pp. - -, therefor.
93	P	Page 132 Specification (10/10/1996)	30	76	67	After "below" delete "pGPla".
94	A	Page 136 Specification (10/10/1996)	27	79	49	Delete "deleteon" and insert - - deletion - -, therefor.
95	P	Page 138 Specification (10/10/1996)	12	80	52	Delete "ou" and insert - - $\sigma\mu$ - -, therefor.
96	A	Page 170 Specification (10/10/1996)	32	101	57	Delete "immmunoglbulins" and insert - - immunoglobulins - -, therefor.
97	P	Page 172 Specification (10/10/1996)	10	102	46	Delete "pNNO3." and insert - - pNN03. - -, therefor.
98	A	Page 176 Specification (10/10/1996)	26	105	4	Delete "(Cyrstal" and insert - - (Crystal - -, therefor.
99	A	Page 180 Specification (10/10/1996)	21	107	27	Delete "e.g." and insert - - e.g., - -, therefor.
100	P	Page 180 Specification (10/10/1996)	30	108	8	Delete "1-50," and insert - - 10-50, - -, therefor.
101	P	Page 182 Specification (10/10/1996)	21	108	37	Delete "West" and insert - - Westr - -, therefor.
102	P	Page 184 Specification (10/10/1996)	5	109	32	Delete "Of" and insert - - of - -, therefor.
103	P	Page 184 Specification (10/10/1996)	34	109	63	After "mouse μ" insert - - and - -.
104	P	Page 185 Specification (10/10/1996)	22	110	23	Delete "IgX" and insert - - Igλ - -, therefor.
105	A	Page 186 Specification (10/10/1996)	27	110	65	Delete "intergration" and insert - - integration - -, therefor.
106	A	Page 186 Specification (10/10/1996)	31	111	2	Delete "intergration" and insert - - integration - -, therefor.
107	A	Page 187 Specification (10/10/1996)	16	111	25-26	Delete "immmunoglobulin" and insert - - immunoglobulin - -, therefor.
108	A	Page 187 Specification (10/10/1996)	27	111	37	Delete "immmunoglobulin" and insert - - immunoglobulin - -, therefor.

109	A	Page 191 Specification (10/10/1996)	18	113	56	Delete "(i.e," and insert - - (i.e., - -, therefor.
110	A	Page 191 Specification (10/10/1996)	21	113	59	Delete "(i.e," and insert - - (i.e., - -, therefor.
111	A	Page 194 Specification (10/10/1996)	32	115	57	Delete "reconstitutued" and insert - - reconstituted - -, therefor.
112	A	Page 196 Specification (10/10/1996)	38	117	8	Delete "phosphotransferse" and insert - - phosphotransferase - -, therefor.
113	P	Page 203 Specification (10/10/1996)	8	120	53	Delete "J_H3," and insert - - JH3, - -, therefor.
114	P	Page 203 Specification (10/10/1996)	9	120	54	Delete "Eu, Su," and insert - - Eμ, Sμ, - -, therefor.
115	P	Page 207 Specification (10/10/1996)	21	123	29	Delete "IgX" and insert - - Igλ - -, therefor.
116	A	Page 209 Specification (10/10/1996)	27	124	45	Delete "Trans-switching" and insert - - Trans-Switching - -, therefor.
117	A	Page 213 Specification (10/10/1996)	3	126	42	Delete "seqeunce" and insert - - sequence - -, therefor.
118	P	Page 216 Specification (10/10/1996)	13	128	7	Delete "31" and insert - - 3' - -, therefor.
119	P	Page 216 Specification (10/10/1996)	16	128	10	Delete "31" and insert - - 3' - -, therefor.
120	P	Page 216 Specification (10/10/1996)	17	128	12	Delete "31" and insert - - 3' - -, therefor.
121	P	Page 218 Specification (10/10/1996)	8	129	2	Delete "Igu" and insert - - Igμ - -, therefor.
122	P	Page 218 Specification (10/10/1996)	10	129	3	Delete "Igu" and insert - - Igμ - -, therefor.
123	P	Page 221 Specification (10/10/1996)	12	130	62	Delete "IgEK" and insert - - IgEκ - -, therefor.
124	P	Page 221 Specification (10/10/1996)	14	130	64	Delete "IgEK" and insert - - IgEκ - -, therefor.
125	P	Page 221 Specification (10/10/1996)	17	130	67	Delete "λCoated Plates" and insert - - λ Coated Plates. - -, therefor.
126	A	Page 221 Specification (10/10/1996)	25	131	9	After "IgG" insert - - . - -.

127	P	Page 222 Specification (10/10/1996)	30	131	54	Delete "Anti-human" and insert - - Anti-Human - -, therefor.
128	P	Page 224 Specification (10/10/1996)	22	132	60	Delete "u" and insert - - μ - -, therefor.
129	P	Page 224 Specification (10/10/1996)	27	132	64	Delete "u" and insert - - μ - -, therefor.
130	A	Page 225 Specification (10/10/1996)	36	133	51	Delete "inununoglobuhn" and insert - - immunoglobulin - -, therefor.
131	P	Page 224 Specification (10/10/1996)	22	134	58	Delete "V_H" and insert - - VH - -, therefor.
132	P	Page 228 Specification (10/10/1996)	2	135	3	Delete "K" and insert - - κ - -, therefor.
133	P	Page 237 Specification (10/10/1996)	28-29	140	34	Delete "15 ng" and insert - - 15 ng/ml, - -, therefor.
134	P	Page 245 Specification (10/10/1996)	17	144	28	Delete "$B202^{bright}$" and insert - - $B220^{bright}$ - -, therefor.
135	P	Page 247 Specification (10/10/1996)	4-24	145	27-46	Delete "Wells.....(ABT). For the.....(Jackson). Murine.....205). Appropriate antibodies.....followed." and insert the same on line 26 as a continuation of paragraph.
136	P	Page 248 Specification (10/10/1996)	1 (Table 16)	146	30	Delete "Eor" and insert - - For - -, therefor.
137	A	Page 249 Specification (10/10/1996)	22	146	57	Delete "absorbtivity" and insert - - absorptivity - -, therefor.
138	P	Page 252 Specification (10/10/1996)	12	148	42	Delete "calorimetric" and insert - - colorimetric - -, therefor.

US007084260B1

(12) **United States Patent**
Lonberg et al.

(10) **Patent No.: US 7,084,260 B1**
(45) **Date of Patent: Aug. 1, 2006**

(54) **HIGH AFFINITY HUMAN ANTIBODIES AND HUMAN ANTIBODIES AGAINST HUMAN ANTIGENS**

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(51) **Int. Cl.**
C07K 16/00 (2006.01)

(52) **U.S. Cl.** **530/388.75**; 530/387.3; 530/388.1

(58) **Field of Classification Search** 435/69.1, 435/69.6, 440, 455, 332, 343.1, 387.3, 388.1; 530/387.1, 388.22, 388.75, 388.15; 536/23.1, 536/23.53; 800/4, 6
See application file for complete search history.

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(74) *Attorney, Agent, or Firm*—Darby & Darby P.C.

(57) **ABSTRACT**

The invention relates to transgenic non-human animals capable of producing high affinity human sequence antibodies. The invention is also directed to human sequence antibodies specific for human antigens, such as, human CD4. The invention also is directed to methods for producing human sequence antibodies.

7 Claims, 99 Drawing Sheets

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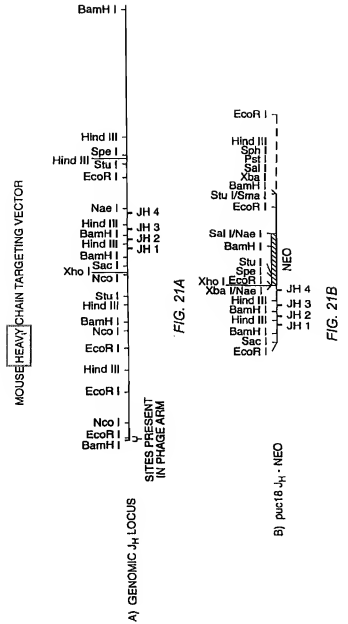
MOUSE HEAVY CHAIN TARGETING VECTOR
BamH I
Hind III
Spe I
Hind III Stu I
EcoR I
Nae I
JH 4
Hind III
BamH I
JH 3
Hind III
JH 2
BamH I
JH 1
Xho I Sac I
Nco I
Stu I
Hind III
BamH I
Nco I
EcoR I
Hind III
EcoR I
Nco I
EcoR I
BamH I
SITES PRESENT IN PHAGE ARM
A) GENOMIC J_H LOCUS
FIG. 21A
EcoR I
Hind III
Sph I
Pst I
Sal I
Xba I
BamH I
Stu I/Sma I
EcoR I
Sal I/Nae I
BamH I
NEO
Stu I
Spe I
Xho I EcoR I
Xba I/Nae I
JH 4
Hind III
BamH I
JH 3
Hind III
JH 2
BamH I
JH 1
Sac I
EcoR I
B) puc18 J_H - NEO
FIG. 21B
Not I
Sal I
Xho I
Xba I
EcoR I
TK
Hind III
Not I
C) pGMT-TK
FIG. 21C

HIGH AFFINITY HUMAN ANTIBODIES AND HUMAN ANTIBODIES AGAINST HUMAN ANTIGENS

TECHNICAL FIELD

The invention relates to transgenic non-human animals capable of producing heterologous antibodies, transgenes used to produce such transgenic animals, transgenes capable of functionally rearranging a heterologous D gene in V-D-J recombination, immortalized B-cells capable of producing heterologous antibodies, methods and transgenes for producing heterologous antibodies of multiple isotypes, methods and transgenes for producing heterologous antibodies wherein a variable region sequence comprises somatic mutation as compared to *germline* rearranged variable region sequences, transgenic nonhuman animals which produce antibodies having a human primary sequence and which bind to human antigens, hybridomas made from B cells of such transgenic animals, and monclonal antibodies expressed by such hybridomas.

BACKGROUND OF THE INVENTION

One of the major impediments facing the development of in vivo therapeutic and diagnostic applications for monoclonal antibodies in humans is the intrinsic immunogenicity of non-human immunoglobulins. For example, when immunocompetent human patients are administered therapeutic doses of rodent monoclonal antibodies, the patients produce antibodies against the rodent immunoglobulin sequences; these human anti-mouse antibodies (HAMA) neutralize the therapeutic antibodies and can cause acute toxicity. Hence, it is desirable to produce human immunoglobulins that are reactive with specific human antigens that are promising therapeutic and/or diagnostic targets. However, producing human immunoglobulins that bind specifically with human antigens is problematic.

The present technology for generating monoclonal antibodies involves pre-exposing, or priming, an animal (usually a rat or mouse) with antigen, harvesting B-cells from that animal, and generating a library of hybridoma clones. By screening a hybridoma population for antigen binding specificity (idiotype) and also screening for immunoglobulin class (isotype), it is possible to select hybridoma clones that secrete the desired antibody.

However, when present methods for generating monoclonal antibodies are applied for the purpose of generating human antibodies that have binding specificities for human antigens, obtaining B-lymphocytes which produce human immunoglobulins a serious obstacle, since humans will typically not make immune responses against self-antigens.

Hence, present methods of generating human monoclonal antibodies that are specifically reactive with human antigens are clearly insufficient. It is evident that the same limitations on generating monoclonal antibodies to authentic self antigens apply where non-human species are used as the source of B-cells for making the hybridoma.

The construction of transgenic animals harboring a functional heterologous immunoglobulin transgene are a method by which antibodies reactive with self antigens may be produced. However, in order to obtain expression of therapeutically useful antibodies, or hybridoma clones producing such antibodies, the transgenic animal must produce transgenic B cells that are capable of maturing through the B lymphocyte development pathway. Such maturation requires the presence of surface IgM on the transgenic B cells, however isotypes other than IgM are desired for therapeutic uses. Thus, there is a need for transgenes and animals harboring such transgenes that are able to undergo functional V-D-J rearrangement to generate recombinational diversity and junctional diversity. Further, such transgenes and transgenic animals preferably include cis-acting sequences that facilitate isotype switching from a first isotype that is required for B cell maturation to a subsequent isotype that has superior therapeutic utility.

A number of experiments have reported the use of transfected cell lines to determine the specific DNA sequences required for Ig gene rearrangement (reviewed by Lewis and Gellert (1989), *Cell*, 59, 585–588). Such reports have identified putative sequences and concluded that the accessibility of these sequences to the recombinase enzymes used for rearrangement is modulated by transcription (Yancopoulos and Alt (1985), *Cell*, 40, 271–281). The sequences for V(D)J joining are reportedly a highly conserved, near-palindromic heptamer and a less well conserved AT-rich nanomer separated by a spacer of either 12 or 23 bp (Tonegawa (1983), *Nature*, 302, 575–581; Hesse, et al. (1989), *Genes in Dev.*, 3, 1053–1061). Efficient recombination reportedly occurs only between sites containing recombination signal sequences with different length spacer regions.

Ig gene rearrangement, though studied in tissue culture cells, has not been extensively examined in transgenic mice. Only a handful of reports have been published describing rearrangement test constructs introduced into mice [Buchini, et al. (1987), *Nature*, 326, 409–411 (unrearranged chicken λ transgene); Goodhart, et al. (1987), *Proc. Natl. Acad. Sci. USA*, 84, 4229–4233) (unrearranged rabbit κ gene); and Bruggemann, et al. (1989), *Proc. Natl. Acad. Sci. USA*, 86, 6709–6713 (hybrid mouse-human heavy chain)]. The results of such experiments, however, have been variable, in some cases, producing incomplete or minimal rearrangement of the transgene.

Further, a variety of biological functions of antibody molecules are exerted by the Fc portion of molecules, such as the interaction with mast cells or basophils through Fcε, and binding of complement by Fcμ or Fcγ, it further is desirable to generate a functional diversity of antibodies of a given specificity by variation of isotype.

Although transgenic animals have been generated that incorporate transgenes encoding one or more chains of a heterologous antibody, there have been no reports of heterologous transgenes that undergo successful isotype switching. Transgenic animals that cannot switch isotypes are limited to producing heterologous antibodies of a single isotype, and more specifically are limited to producing an isotype that is essential for B cell maturation, such as IgM and possibly IgD, which may be of limited therapeutic utility. Thus, there is a need for heterologous immunoglobulin transgenes and transgenic animals that are capable of switching from an isotype needed for B cell development to an isotype that has a desired characteristic for therapeutic use.

Based on the foregoing, it is clear that a need exists for methods of efficiently producing heterologous antibodies, e.g. antibodies encoded by genetic sequences of a first species that are produced in a second species. More particularly, there is a need in the art for heterologous immunoglobulin transgenes and transgenic animals that are capable of undergoing functional V-D-J gene rearrangement that incorporates all or a portion of a D gene segment which contributes to recombinational diversity. Further, there is a need in the art for transgenes and transgenic animals that can

will typically be a function of the origin of the associated switch regions. Alternatively, or in combination with switch sequences, δ-associated deletion sequences may be linked to various C_H genes to effect non-classical switching by deletion of sequences between two δ-associated deletion sequences. Thus, a transgene may be constructed so that a particular C_H gene is linked to a different switch sequence and thereby is switched to more frequently than occurs when the naturally associated switch region is used.

This invention also provides methods to determine whether isotype switching of transgene sequences has occurred in a transgenic animal containing an immunoglobulin transgene.

The invention provides immunoglobulin transgene constructs and methods for producing immunoglobulin transgene constructs, some of which contain a subset of germline immunoglobulin loci sequences (which may include deletions). The invention includes a specific method for facilitated cloning and construction of immunoglobulin transgenes, involving a vector that employs unique XhoI and SalI restriction sites flanked by two unique NotI sites. This method exploits the complementary termini of XhoI and SalI restrictions sites and is useful for creating large constructs by ordered concatemerization of restriction fragments in a vector.

The transgenes of the invention include a heavy chain transgene comprising DNA encoding at least one variable gene segment, one diversity gene segment, one joining gene segment and one constant region gene segment. The immunoglobulin light chain transgene comprises DNA encoding at least one variable gene segment, one joining gene segment and one constant region gene segment. The gene segments encoding the light and heavy chain gene segments are heterologous to the transgenic non-human animal in that they are derived from, or correspond to, DNA encoding immunoglobulin heavy and light chain gene segments from a species not consisting of the transgenic non-human animal. In one aspect of the invention, the transgene is constructed such that the individual gene segments are unrearranged, i.e., not rearranged so as to encode a functional immunoglobulin light or heavy chain. Such unrearranged transgenes permit recombination of the gene segments (functional rearrangement) and expression of the resultant rearranged immunoglobulin heavy and/or light chains within the transgenic non-human animal when said animal is exposed to antigen.

In one aspect of the invention, heterologous heavy and light immunoglobulin transgenes comprise relatively large fragments of unrearranged heterologous DNA. Such fragments typically comprise a substantial portion of the C, J (and in the case of heavy chain, D) segments from a heterologous immunoglobulin locus. In addition, such fragments also comprise a substantial portion of the variable gene segments.

In one embodiment, such transgene constructs comprise regulatory sequences, e.g., promoters, enhancers, class switch regions, recombination signals and the like, corresponding to sequences derived from the heterologous DNA. Alternatively, such regulatory sequences may be incorporated into the transgene from the same or a related species of the non-human animal used in the invention. For example, human immunoglobulin gene segments may be combined in a transgene with a rodent immunoglobulin enhancer sequence for use in a transgenic mouse.

In a method of the invention, a transgenic non-human animal containing germline unrearranged light and heavy immunoglobulin transgenes—that undergo VDJ joining during D-cell differentiation—is contacted with an antigen to induce production of a heterologous antibody in a secondary repertoire B-cell.

Also included in the invention are vectors and methods to disrupt the endogenous immunoglobulin loci in the non-human animal to be used in the invention. Such vectors and methods utilize a transgene, preferably positive-negative selection vector, which is constructed such that it targets the functional disruption of a class of gene segments encoding a heavy and/or light immunoglobulin chain endogenous to the non-human animal used in the invention. Such endogenous gene segments include diversity, joining and constant region gene segments. In this aspect of the invention, the positive-negative selection vector is contacted with at least one embryonic stem cell of a non-human animal after which cells are selected wherein the positive-negative selection vector has integrated into the genome of the non-human animal by way of homologous recombination. After transplantation, the resultant transgenic non-human animal is substantially incapable of mounting an immunoglobulin-mediated immune response as a result of homologous integration of the vector into chromosomal DNA. Such immune deficient non-human animals may thereafter be used for study of immune deficiencies or used as the recipient of heterologous immunoglobulin heavy and light chain transgenes.

The invention also provides vectors, methods, and compositions useful for suppressing the expression of one or more species of immunoglobulin chain(s), without disrupting an endogenous immunoglobulin locus. Such methods are useful for suppressing expression of one or more endogenous immunoglobulin chains while permitting the expression of one or more transgene-encoded immunoglobulin chains. Unlike genetic disruption of an endogenous immunoglobulin chain locus, suppression of immunoglobulin chain expression does not require the time-consuming breeding that is needed to establish transgenic animals homozygous for a disrupted endogenous Ig locus. An additional advantage of suppression as compared to engognous Ig gene disruption is that, in certain embodiments, chain suppression is reversible within an individual animal. For example, Ig chain suppression may be accomplished with: (1) transgenes encoding and expressing antisense RNA that specifically hybridizes to an endogenous Ig chain gene sequence, (2) antisense oligonucleotides that specifically hybridize to an endogenous Ig chain gene sequence, and (3) immunoglobulins that bind specifically to an endogenous Ig chain polypeptide.

The invention provides transgenic non-human animals comprising: a homozygous pair of functionally disrupted endogenous heavy chain alleles, a homozygous pair of functionally disrupted endogenous light chain alleles, at least one copy of a heterologous immunoglobulin heavy chain transgene, and at least one copy of a heterologous immunoglobulin heavy chain transgene, wherein said animal makes an antibody response following immunization with an antigen, such as a human antigen (e.g., CD4). The invention also provides such a transgenic non-human animal wherein said functionally disrupted endogenous heavy chain allele is a J_H region homologous recombination knockout, said functionally disrupted endogenous light chain allele is a $J_κ$ region homologous recombination knockout, said heterologous immunoglobulin heavy chain transgene is the HC1 or HC2 human minigene transgenes, said heterologous light chain transgene is the KC2 or KC1e human κ transgene, and wherein said antigen is a human antigen.

The invention also provides various embodiments for suppressing, ablating, and/or functionally disrupting the endogenous nonhuman immunoglobulin loci.

The invention also provides transgenic mice expressing both human sequence heavy chains and chimeric heavy chains comprising a human sequence heavy chain variable region and a murine sequence heavy chain constant region. Such chimeric heavy chains are generally produced by trans-switching between a functionally rearranged human transgene and an endogenous murine heavy chain constant region (e.g., γ1, γ2a, γ2b, γ3). Antibodies comprising such chimeric heavy chains, typically in combination with a transgene-encoded human sequence light chain or endogenous murine light chain, are formed in response to immunization with a predetermined antigen. The transgenic mice of these embodiments can comprise B cells which produce (express) a human sequence heavy chain at a first timepoint and trans-switch to produce (express) a chimeric heavy chain composed of a human variable region and a murine constant region (e.g., γ1, γ2a, γ2b, γ3) at a second (subsequent) timepoint; such human sequence and chimeric heavy chains are incorporated into functional antibodies with light chains; such antibodies are present in the serum of such transgenic mice. Thus, to restate: the transgenic mice of these embodiments can comprise B cells which express a human sequence heavy chain and subsequently switch (via trans-switching or cis-switching) to express a chimeric or isotype-switched heavy chain composed of a human variable region and a alternative constant region (e.g., murine γ1, γ2a, γ2b, γ3; human γ, α, ε); such human sequence and chimeric or isotype-switched heavy chains are incorporated into functional antibodies with light chains (human or mouse); such antibodies are present in the serum of such transgenic mice.

The invention also provides a method for generating a large transgene, said method comprising:

introducing into a mammalian cell at least three polynucleotide species; a first polynucleotide species having a recombinogenic region of sequence identity shared with a second polynucleotide species, a second polynucleotide species having a recombinogenic region of sequence identity shared with a first polynucleotide species and a recombinogenic region of sequence identity shared with a third polynucleotide species, and a third polynucleotide species having a recombinogenic region of sequence identity shared with said second polynucleotide species.

Recombinogenic regions are regions of substantial sequence identity sufficient to generate homologous recombination in vivo in a mammalian cell (e.g., ES cell), and preferably also in non-mammalian eukaryotic cells (e.g., Saccharaomyces and other yeast or fungal cells). Typically, recombinogenic regions are at least 50 to 100000 nucleotides long or longer, preferably 500 nucleotides to 10000 nucleotides long, and are often 80–100 percent identical, frequently 95–100 percent identical, often isogenic.

The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** depicts the complementarity determining regions CDR1, CDR2 and CDR3 and framework regions FR1, FR2, FR3 and FR4 in unrearranged genomic DNA and mRNA expressed from a rearranged immunoglobulin heavy chain gene,

FIG. **2** depicts the human λ chain locus,

FIG. **3** depicts the human κ chain locus,

FIG. **4** depicts the human heavy chain locus,

FIG. **5** depicts a transgene construct containing a rearranged IgM gene ligated to a 25 kb fragment that contains human γ3 and γ1 constant regions followed by a 700 bp fragment containing the rat chain 3' enhancer sequence.

FIG. **6** is a restriction map of the human κ chain locus depicting the fragments to be used to form a light chain transgene by way of in vivo homologous recombination.

FIG. **7** depicts the construction of pGP1.

FIG. **8** depicts the construction of the polylinker contained in pGP1.

FIG. **9** depicts the fragments used to construct a human heavy chain transgene of the invention.

FIG. **10** depicts the construction of pHIG1 and pCON1.

FIG. **11** depicts the human Cγ1 fragments which are inserted into pRE3 (rat enhancer 3') to form pREG2.

FIG. **12** depicts the construction of pHIG3' and PCON.

FIG. **13** depicts the fragment containing human D region segments used in construction of the transgenes of the invention.

FIG. **14** depicts the construction of pHIG2 (D segment containing plasmid).

FIG. **15** depicts the fragments covering the human Jκ and human Cκ gene segments used in constructing a transgene of the invention.

FIG. **16** depicts the structure of pJμ.

FIG. **17** depicts the construction of pKapH.

FIGS. **18**A through **18**D depict the construction of a positive-negative selection vector for functionally disrupting the endogenous heavy chain immunoglobulin locus of mouse.

FIGS. **19**A through **19**C depict the construction of a positive-negative selection vector for functionally disrupting the endogenous immunoglobulin light chain loci in mouse.

FIGS. **20**A through **20**E depict the structure of a kappa light chain targeting vector.

FIGS. **21**A through **21**F depict the structure of a mouse heavy chain targeting vector.

FIG. **22** depicts the map of vector pGPe.

FIG. **23** depicts the structure of vector pJM2.

FIG. **24** depicts the structure of vector pCOR1.

FIG. **25** depicts the transgene constructs for pIGM1, pHC1 and pHC2.

FIG. **26** depicts the structure of pγe2.

FIG. **27** depicts the structure of pVGE1.

FIG. **28** depicts the assay results of human Ig expression in a pHC1 transgenic mouse.

FIG. **29** depicts the structure of pJCK1.

FIG. **30** depicts the construction of a synthetic heavy chain variable region.

FIG. **31** is a schematic representation of the heavy chain minilocus constructs pIGM$_1$, pHC1, and pHC2.

FIG. **32** is a schematic representation of the heavy chain minilocus construct pIGG1 and the κ light chain minilocus construct pKC1, pKVe1, and pKC2.

FIG. **33** depicts a scheme to reconstruct functionally rearranged light chain genes.

FIG. **34** depicts serum ELISA results

FIG. **35** depicts the results of an ELISA assay of serum from 8 transgenic mice.

FIG. **36** is a schematic representation of plasmid pBCE1.

FIGS. **37**A–**37**C depict the immune response of transgenic mice of the present invention against KLH-DNP, by measuring IgG and IgM levels specific for KLH-DNP (37A), KLH (37B) and BSA-DNP (37C).

FIG. **38** shows ELISA data demonstrating the presence of antibodies that bind human carcinoembryonic antigen (CEA) and comprise human μ chains; each panel shows reciprocal serial dilutions from pooled serum samples obtained from mice on the indicated day following immunization.

FIG. **39** shows ELISA data demonstrating the presence of antibodies that bind human carcinoembryonic antigen (CEA) and comprise human γ chains; each panel shows reciprocal serial dilutions from pooled serum samples obtained from mice on the indicated day following immunization.

FIG. **40** shows aligned variable region sequences of 23 randomly-chosen cDNAs (SEQ ID NOS:271, 273, **274**, 276–289, 291, 292 and 294–297) generated from mRNA obtained from lymphoid tissue of HC1 transgenic mice immunized with human carcinoembryonic antigen (CEA) as compared to the germline transgene sequence (top line) (SEQ ID NO:269), on each line nucleotide changes relative to germline sequence are shown. The regions corresponding to heavy chain CDR1, CDR2, and CDR3 are indicated. Non-germline encoded nucleotides are shown in capital letters. J segments=SEQ ID NOS:270, 272, 275, 290 and 293.

FIG. **41** shows the nucleotide sequence of a human DNA fragment, designated vk65.3, containing a V_κ gene segment (SEQ ID NO:298); the deduced amino acid sequences of the V_κ coding regions are also shown (SEQ ID NO:299); splicing and recombination signal sequences (heptamer/nonamer) are shown boxed.

FIG. **42** shows the nucleotide sequence of a human DNA fragment, designated vk65.5, containing a V_κ gene segment (SEQ ID NO:300); the deduced amino acid sequences of the V_κ coding regions are also shown(SEQ ID NO:301); splicing and recombination signal sequences (heptamer/nonamer) are shown boxed.

FIG. **43** shows the nucleotide sequence of a human DNA fragment, designated vk65.8, containing a V_κ gene segment (SEQ ID NO:302); the deduced amino acid sequences of the V_κ coding regions are also shown (SEQ ID NO:303); splicing and recombination signal sequences (heptamer/nonamer) are shown boxed.

FIG. **44** shows the nucleotide sequence of a human DNA fragment, designated vk65.15, containing a V_κ gene segment(SEQ ID NO:304); the deduced amino acid sequences of the V_κ coding regions are also shown(SEQ ID NO:305); splicing and recombination signal sequences (heptamer/nonamer) are shown boxed.

FIG. **45** shows formation of a light chain minilocus by homologous recombination between two overlapping fragments which were co-injected.

FIG. **46** shows ELISA results for monoclonal antibodies reactive with CEA and non-CEA antigens showing the specificity of antigen binding.

FIG. **47** shows the DNA sequences of 10 cDNAs (SEQ ID NOS:306–315) amplified by PCR to amplify transcripts having a human VDJ and a murine constant region sequence.

FIG. **48** shows ELISA results for various dilutions of serum obtained from mice bearing both a human heavy chain minilocus transgene and a human κ minilocus transgene, the mouse was immunized with human CD4 and the data shown represents antibodies reactive with human CD4 and possessing human κ, human μ, or human γ epitopes, respectively.

FIG. **49** shows relative distribution of lymphocytes staining for human μ or mouse μ as determined by FACS for three mouse genotypes.

FIG. **50** shows relative distribution of lymphocytes staining for human κ or mouse κ as determined by FACS for three mouse genotypes.

FIG. **51** shows relative distribution of lymphocytes staining for mouse λ as determined by FACS for three mouse genotypes.

FIG. **52** shows relative distribution of lymphocytes staining for mouse λ or human κ as determined by FACS for four mouse genotypes.

FIG. **53** shows the amounts of human μ, human γ, human κ, mouse μ, mouse γ, mouse κ, and mouse λ chains in the serum of unimmunized 0011 mice.

FIG. **54** shows a scatter plot showing the amounts of human μ, human γ, human κ, mouse μ, mouse γ, mouse κ, and mouse λ chains in the serum of unimmunized 0011 mice of various genotypes.

FIG. **55** shows the titres of antibodies comprising human μ, human γ, or human κ chains in anti-CD4 antibodies in the serum taken at three weeks or seven weeks post-immunization following immunization of a 0011 mouse with human CD4.

FIG. **56** shows a schematic representation of the human heavy chain minilocus transgenes pHC1 and pHC2, and the light chain minilocus transgenes pKC1, pKC1e, and the light chain minilocus transgene created by homologous recombination between pKC2 and Co4 at the site indicated.

FIG. **57** shows a linkage map of the murine lambda light chain locus as taken from Storb et al. (1989) *op.cit.*; the stippled boxes represent a pseudogene.

FIG. **58** shows a schematic representation of inactivation of the murine λ locus by homologous gene targeting.

FIG. **59** schematically shows the structure of a homologous recombination targeting transgene for deleting genes, such as heavy chain constant region genes.

FIG. **60** shows a map of the BALB/c murine heavy chain locus as taken from *Immunoglobulin Genes*, Honjo, T, Alt, F W, and Rabbits T H (eds.) Academic Press, NY (1989) p. 129. Structural genes are shown by closed boxes in the top line; second and third lines show restriction sites with symbols indicated.

FIG. **61** shows a nucleotide sequence (SEQ ID NO:316) of mouse heavy chain locus α constant region gene.

FIG. **62** shows the construction of a frameshift vector (plasmid B) for introducing a two bp framcshift into the murine heavy chain locus J_4 gene.

FIG. **63** shows isotype specific response of transgenic animals during hyperimmunization. The relative levels of reactive human μ and γ1 are indicated by a calorimetric ELISA assay (y-axis). We immunized three 7–10 week old male HC1 line 57 transgenic animals (#1991, #2356, #2357), in a homozygous JHD background, by intraperitoneal injections of CEA in Freund's adjuvant. The figure depicts binding of 250 fold dilutions of pooled serum (collected prior to each injection) to CEA coated microtiter wells.

FIGS. **64**A and **64**B show expression of transgene encoded γ1 isotype mediated by class switch recombination.

propidium iodide staining and light scatter. The fraction of B220+ cells in each of the samples displayed in the bottom row is given as a percent of the lymphocyte light scatter gate.

FIG. **70**. Secreted immunoglobulin levels in the serum of double transgenic mice. Human μ, γ, and κ, and mouse γ and λ from 18 individual HC2/KCo4 double transgenic mice homozygous for endogenous heavy and κ-light chain locus disruption. Mice: (+) HC2 line 2550 (~5 copies of HC2 per integration), KCo4 line 4436 (1–2 copies of KCo4 per integration); (○) HC2 line 2550, KCo4 line 4437 (~10 copies of KCo4 per integration); (x) HC2 line 2550, KCo4 line 4583 (~5 copies of KCo4 per integration); (□) HC2 line 2572 (30–50 copies of HC2 per integration, KCo4 line 4437; ▯ (..) HC2 line 5467 (20–30 copies of HC2 per integration, KCo4 line 4437).

FIGS. **71**A and **71**B show human antibody responses to human antigens. FIG. **71**A: Primary response to recombinant human soluble CD4. Levels of human IgM and human κ light chain are reported for prebleed (○) and post-immunization (●) serum from four double transgenic mice. FIG. **71**B: Switching to human IgG occurs in vivo. Human IgG (circles) was detected with peroxidase conjugated polyclonal anti-human IgG used in the presence of 1.5 μ/ml excess IgE, κ and 1% normal mouse serum to inhibit non-specific cross-reactivity. Human κ light chain (squares) was detected using a peroxidase conjugated polyclonal anti-human κ reagent in the presence of 1% normal mouse serum. A representative result from one mouse (#9344; HC2 line 2550, KCo4 line 4436) is shown. Each point represents an average of duplicate wells minus background absorbance.

FIG. **72** shows FACS analysis of human PBL with a hybridoma supernatant that discriminates human CD4+ lymphocytes from human CD8+ lymphocytes.

FIG. **73** shows human α-CD4 IgM anf IgG in transgenic mouse serum.

FIG. **74** shows competition binding experiments comparing a transgenic mouse α-human CD4 hybridoma monoclonal, 2C11-8, to the RPA-TA and Leu-3A monoclonals.

FIG. **75** shows production data for Ig expression of cultured 2C11-8 hybridoma.

FIG. **76** shows an overlapping set of plasmid inserts constituting the HCo7 transgene.

FIG. **77**A depicts the nucleotide sequence (SEQ ID NO:409) of pGP2b plasmid vector.

FIG. **77**B depicts the restriction map of pGP2b plasmid vector.

FIG. **78** (parts A and B) depicts cloning strategy for assembling large transgenes.

FIG. **79** shows that large inserts are unstable in high-copy pUC derived plasmids.

FIG. **80** shows phage P1 clone P1-570. Insert spans portion of human heavy chain constant region covering γ3 and γ1, together with switch elements. N, NotI; S, SalI, X, XhoI.

FIG. **81** shows serum expression of human μ and γ1 in HCo7 transgenic founder animals.

FIG. **82** shows serum expression of human immunoglobulins in HCo7/KCo4 double transgenic/double deletion mice.

FIG. **83** shows RT PCR detection of human γ1 and γ3 transcripts in HCo7 transgenic mouse spleen RNA.

FIG. **84** shows induction of human IgG1 and IgG3 by LPS and IL-4 in vitro.

FIG. **85**. Agarose gel electrophoresis apparatus for concentration of YAC DNA.

FIG. **86**. Two color FACS analysis of bone marrow cells from HC2/KCo5/JHD/JKD and HC2/KCo4/JHD/JKD mice. The fraction of cells in each of the B220$^+$/CD43$^-$, B220$^+$/CD43$^+$, and B220$^+$/IgM$^+$ gates is given as a percent.

FIG. **87**. Two color FACS analysis of spleen cells from HC2/KCo5/JHD/JKD and HC2/KCo4/JHD/JKD mice. The fraction of cells in each of the B220^{bright}/IgM$^+$ and B220^{dull}/IgM$^+$ gates is given as a percent.

FIG. **88**. Binding of IgGκ anti-nCD4 monoclonal antibodies to CD4+ SupT1 cells.

FIG. **89** Epitope determination for IgG anti-nCD4 monoclonal antibodies by flow cytometry. SupT1 cells were pre-incubated with buffer (left column), 2.5 mg/ml RPA-T4 (middle column), or 2.5 mg/ml Leu3a (right column) and then with one of the 10 human IgG monoclonal antibodies (in supernatant diluted 1:2), or chimeric Leu3a. Results for 3 representative human IgG monoclonal antibodies are shown in this figure.

FIG. **90** Inhibition of an MLR by a human IgGκ anti-CD4 monoclonal antibody.

Table 1 depicts the sequence of vector pGPc.

Table 2 depicts the sequence of gene $V_H49.8$.

Table 3 depicts the detection of human IgM and IgG in the serum of transgenic mice of this invention.

Table 4 depicts sequences of VDJ joints.

Table 5 depicts the distribution of J segments incorporated into pHC1 transgene encoded transcripts to J segments found in adult human peripheral blood lymphocytes (PBL).

Table 6 depicts the distribution of D segments incorporated into pHC1 transgene encoded transcripts to D segments found in adult human peripheral blood lymphocytes (PBL).

Table **7** depicts the predicted amino acid sequences of the VDJ regions from 30 clones analyzed from pHC1 transgenic.

Table 8 depicts the length of the CDR3 peptides from transcripts with in-frame VDJ joints in the pHC1 transgenic mouse and in human PBL.

Table 9 shows transgenic mice of line 112 that were used in the indicated experiments; (+) indicates the presence of the respective transgene, (++) indicates that the animal is homozygous for the J_HD knockout transgene.

Table **10** shows the genotypes of several 0011 mice.

Table 11 shows human variable region usage in hybridomas from transgenic mice.

Table 12 shows transgene V and J segment usage.

Table 13 shows the occurrence of somatic mutation in the HC2 heavy chain transgene in transgenic mice.

Table 14 shows identification of human $V_κ$ segments on the YAC 4x17B1.

Table 15. Identification of human Vk genes expressed in mouse line KCo5-9272.

Table 16. Secretion levels for human IgGk Anti-nCD4 monoclonal antibodies Table 17. Rate and affinity constants for monoclonal antibodies that bind to human CD4.

Table 18. Affinity and rate constants of human anti-human CD4 monoclonal antibodies.

Table 19. Avidity and rate constants of human anti-human CD4 monoclonal antibodies.

Table 20. Avidity and rate constants reported for anti CD4 monoclonal antibodies.

Table 21. Avidity constants of human anti-human CD4 monoclonal antibodies as determined by flow cytometry.

Table 22. Partial Nucleotidc Sequence for Functional Transcripts.

Table 23 Germline V(D)J Segment Usage in Hybridoma Transcripts.

Table 24, Primers, Vectors and Products Used in Minigene Construction.

Table 25. Effect of Human mAbs on Peripheral Chimpanzee Lymphocytcs.

DETAILED DESCRIPTION

As has been discussed supra, it is desirable to produce human immunoglobulins that are reactive with specific human antigens that are promising therapeutic and/or diagnostic targets. However, producing human immunoglobulins that bind specifically with human antigens is problematic.

First, the immunized animal that serves as the source of B cells must make an immune response against the presented antigen. In order for an animal to make an immune response, the antigen presented must be foreign and the animal must not be tolerant to the antigen. Thus, for example, if it is desired to produce a human monoclonal antibody with an idiotype that binds to a human protein, self-tolerance will prevent an immunized human from making a substantial immune response to the human protein, since the only epitopes of the antigen that may be immunogenic will be those that result from polymorphism of the protein within the human population (allogeneic epitopes).

Second, if the animal that serves as the source of B-cells for forming a hybridoma (a human in the illustrative given example) does make an immune response against an authentic self antigen, a severe autoimmune disease may result in the animal. Where humans would be used as a source of B-cells for a hybridoma, such autoimmunization would be considered unethical by contemporary standards. Thus, developing hybridomas secreting human immunoglobulin chainsspecifically reactive with predetermined human antigens is problematic, since a reliable source of human antibody-secreting B cells that can evoke an antibody response against predetermined human antigens is needed.

One methodology that can be used to obtain human antibodies that are specifically reactive with humau antigens is the production of a transgenic mouse harboring the human immunoglobulin transgene constructs of this invention. Briefly, transgenes containing all or portions of the human immunoglobulin heavy and light chain loci, or transgenes containing synthetic "minilocii" (described infra, and in U.S. Ser. No. 08/352,322, filed 7 Dec. 1994 (issued as U.S. Pat. No. 5,625,126 on Apr. 29, 1997), U.S. Ser. No. 07/990,860, filed 16 Dec. 1992 (issued as U.S. Pat. No. 5,545,806 on Aug. 13, 1996), U.S. Ser. No. 07/810,279 filed 17 Dec. 1991 (issued as U.S. Pat. No. 5,569,825 on Oct. 29, 1996), U.S. Ser. No. 07/904,068 filed Jun. 23, 1992 (now abandoned); U.S. Ser. No. 07/853,408, filed 18 Mar. 1992 (issued as U.S. Pat. No. 5,789,650 Aug. 4, 1998), U.S. Ser. No. 07/574,748 filed Aug. 29, 1990 (now abandoned), U.S. Ser. No. 07/575, 962 filed Aug. 31, 1990 (now abandoned), and PCT/US91/06185 filed Aug. 28, 1991, each incorporated herein by reference) which comprise essential functional elements of the human heavy and light chain loci, are employed to produce a transgenic nonhuman animal. Such a transgenic nonhuman animal will have the capacity to produce immunoglobulin chains that are encoded by human immunoglobulin genes, and additionally will be capable of making an immune response against human antigens. Thus, such transgenic animals can serve as a source of immune sera reactive with specified human antigens, and B-cells from such transgenic animals can be fused with myeloma cells to produce hybridomas that secrete monoclonal antibodies that are encoded by human immunoglobulin genes and which are specifically reactive with human antigens.

The production of transgenic mice containing various forms of immunoglobulin genes has been reported previously. Rearranged mouse immunoglobulin heavy or light chain genes have been used to produce transgenic mice. In addition, functionally rearranged human Ig genes including the μ or γ1 constant region have been expressed in transgenic mice. However, experiments in which the transgene comprises unrearranged (V-D-J or V-J not rearranged) immunoglobulin genes have been variable, in some cases, producing incomplete or minimal rearrangement of the transgene. However, there are no published examples of either rearranged or unrearranged immunoglobulin transgenes which undergo successful isotype switching between C_H genes within a transgene.

The invention also provides a method for identifying candidate hybridomas which secrete a monoclonal antibody comprising a human immunoglobulin chain consisting essentially of a human VDJ sequence in polypeptide linkage to a human constant region sequence. Such candidate hybridomas are identified from a pool of hybridoma clones comprising: (1) hybridoma clones that express immunoglobulin chains consisting essentially of a human VDJ region and a human constant region, and (2) trans-switched hybridomas that express heterohybrid immunoglobulin chains consisting essentially of a human VDJ region and a murine constant region. The supernatant(s) of individual or pooled hybridoma clones is contacted with a predetermined antigen, typically an antigen which is immobilized by adsoption onto a solid substrate (e.g., a microtitre well), under binding conditions to select antibodies having the predetermined antigen binding specificity. An antibody that specifically binds to human constant regions is also contacted with the hybridoma supernatant and predetermined antigen under binding conditions so that the antibody selectively binds to at least one human constant region epitope but substantially does not bind to murine constant region epitopes, thus forming complexes consisting essentially of hybridoma supernatant (transgenic monoclonal antibody) bound to a predetermined antigen and to an antibody that specifically binds human constant regions (and which may be labeled with a detectable label or reporter). Detection of the formation of such complexes indicates hybridoma clones or pools which express a human immunoglobulin chain.

In a preferred embodiment of the invention, the anti-human constant region immunoglobulin used in screening specifically recognizes a non-μ, non-δ isotype, preferably a α or ε, more perferrably a γ isotype constant region. Monoclonal antibodies of the γ isotype are preferred (i) because the characteristics of IgG immunoglobulins are preferable to IgM immunoglobulins for some therapeutic applications (e.g., due to the smaller size of the IgG dimers compared to IgM pentamers) and, (ii) because the process of somatic mutation is correlated with the class switch from the μ constant region to the non-μ (e.g., γ) constant regions. Immunoglobulins selected from the population of immunoglobulins that have undergone class switch (e.g., IgG) tend to bind antigen with higher affinity than immunoglobulins selected from the population that has not undergone class switch (e.g., IgM). See, e.g., Lonberg and Huszar. Intern. Rev. Immunol. 13:65–93 (1995) which is incorporated herein by reference.

contemplated (where "derived" indicates that a sequence is identical or modified from another sequence).

A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription regulatory sequences, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

Transgenic Nonhuman Animals Capable of Producing Heterologous Antibodies

The design of a transgenic non-human animal that responds to foreign antigen stimulation with a heterologous antibody repertoire, requires that the heterologous immunoglobulin transgenes contained within the transgenic animal function correctly throughout the pathway of B-cell development. In a preferred embodiment, correct function of a heterologous heavy chain transgene includes isotype switching. Accordingly, the transgenes of the invention are constructed so as to produce isotype switching and one or more of the following: (1) high level and cell-type specific expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

As will be apparent from the following disclosure, not all of the foregoing criteria need be met. For example, in those embodiments wherein the endogenous immunoglobulin loci of the transgenic animal are functionally disrupted, the transgene need not activate allelic exclusion. Further, in those embodiments wherein the transgene comprises a functionally rearranged heavy and/or light chain immunoglobulin gene, the second criteria of functional gene rearrangement is unnecessary, at least for that transgene which is already rearranged. For background on molecular immunology, see, *Fundamental Immunology*, 2nd edition (1989), Paul William E., ed. Raven Press, N.Y., which is incorporated herein by reference.

In one aspect of the invention, transgenic non-human animals are provided that contain rearranged, unrearranged or a combination of rearranged and unrearranged heterologous immunoglobulin heavy and light chain transgenes in the germline of the transgenic animal. Each of the heavy chain transgenes comprises at least one C_H gene. In addition, the heavy chain transgene may contain functional isotype switch sequences, which are capable of supporting isotype switching of a heterologous transgene encoding multiple C_H genes in B-cells of the transgenic animal. Such switch sequences may be those which occur naturally in the germline immunoglobulin locus from the species that serves as the source of the transgene C_H genes, or such switch sequences may be derived from those which occur in the species that is to receive the transgene construct (the transgenic animal). For example, a human transgene construct that is used to produce a transgenic mouse may produce a higher frequency of isotype switching events if it incorporates switch sequences similar to those that occur naturally in the mouse heavy chain locus, as presumably the mouse switch sequences are optimized to function with the mouse switch recombinase enzyme system, whereas the human switch sequences are not. Switch sequences made be isolated and cloned by conventional cloning methods, or may be synthesized de novo from overlapping synthetic oligonucleotides designed on the basis of published sequence information relating to immunoglobulin switch region sequences (Mills et al., *Nucl. Acids Res.* 18:7305–7316 (1991); Sideras et al., *Intl. Immunol.* 1:631–642 (1989), which are incorporated herein by reference).

For each of the foregoing transgenic animals, functionally rearranged heterologous heavy and light chain immunoglobulin transgenes are found in a significant fraction of the B-cells of the transgenic animal (at least 10 percent).

The transgenes of the invention include a heavy chain transgene comprising DNA encoding at least one variable gene segment, one diversity gene segment, one joining gene segment and at least one constant region gene segment. The immunoglobulin light chain transgene comprises DNA encoding at least one variable gene segment, one joining gene segment and at least one constant region gene segment. The gene segments encoding the light and heavy chain gene segments are heterologous to the transgenic non-human animal in that they are derived from, or correspond to, DNA encoding immunoglobulin heavy and light chain gene segments from a species not consisting of the transgenic non-human animal. In one aspect of the invention, the transgene is constructed such that the individual gene segments are unrearranged, i.e., not rearranged so as to encode a functional immunoglobulin light or heavy chain. Such unrearranged transgenes support recombination of the V, D, and J gene segments (functional rearrangement) and preferably support incorporation of all or a portion of a D region gene segment in the resultant rearranged immunoglobulin heavy chain within the transgenic non-human animal when exposed to antigen.

In an alternate embodiment, the transgenes comprise an unrearranged "mini-locus". Such transgenes typically comprise a substantial portion of the C, D, and J segments as well as a subset of the V gene segments. In such transgene constructs, the various regulatory sequences, e.g. promoters, enhancers, class switch regions, splice-donor and splice-acceptor sequences for RNA processing, recombination signals and the like, comprise corresponding sequences derived from the heterologous DNA. Such regulatory sequences may be incorporated into the transgene from the same or a related species of the non-human animal used in the invention. For example, human immunoglobulin gene segments may be combined in a transgene with a rodent immunoglobulin enhancer sequence for use in a transgenic mouse. Alternatively, synthetic regulatory sequences may be incorporated into the transgene, wherein such synthetic regulatory sequences are not homologous to a functional DNA sequence that is known to occur naturally in the genomes of mammals. Synthetic regulatory sequences are designed according to consensus rules, such as, for example, those specifying the permissible sequences of a splice-acceptor site or a promoter/enhancer motif. For example, a minilocus comprises a portion of the genomic immunoglobulin locus having at least one internal (i.e., not at a terminus of the portion) deletion of a non-essential DNA portion (e.g., intervening sequence; intron or portion thereof) as compared to the naturally-occurring germline Ig locus.

The invention also includes transgenic animals containing germ line cells having a heavy and light transgene wherein one of the said transgenes contains rearranged gene segments with the other containing unrearranged gene segments. In the preferred embodiments, the rearranged transgene is a light chain immunoglobulin transgene and the unrearranged transgene is a heavy chain immunoglobulin transgene.

The Structure and Generation of Antibodies

The basic structure of all immunoglobulins is based upon a unit consisting of two light polypeptide chains and two heavy polypeptide chains. Each light chain comprises two regions known as the variable light chain region and the constant light chain region. Similarly, the immunoglobulin heavy chain comprises two regions designated the variable heavy chain region and the constant heavy chain region.

The constant region for the heavy or light chain is encoded by genomic sequences referred to as heavy or light constant region gene (C_H) segments. The use of a particular heavy chain gene segment defines the class of immunoglobulin. For example, in humans, the μ constant region gene segments define the IgM class of antibody whereas the use of a γ1, γ2, γ3 or γ4 constant region gene segment defines the IgG class of antibodies as well as the IgG subclasses IgG1 through IgG4. Similarly, the use of a α_1 or α_2 constant region gene segment defines the IgA class of antibodies as well as the subclasses [illegible]

IgA1 and IgA2. The δ and Σ constant region gene segments define the IgD and IgE antibody classes, respectively.

The variable regions of the heavy and light immunoglobulin chains together contain the antigen binding domain of the antibody. Because of the need for diversity in this region of the antibody to permit binding to a wide range of antigens, the DNA encoding the initial or primary repertoire variable region comprises a number of different DNA segments derived from families of specific variable region gene segments. In the case of the light chain variable region, such families comprise variable (V) gene segments and joining (J) gene segments. Thus, the initial variable region of the light chain is encoded by one V gene segment and one J gene segment each selected from the family of V and J gene segments contained in the genomic DNA of the organism. In the case of the heavy chain variable region, the DNA encoding the initial or primary repertoire variable region of the heavy chain comprises one heavy chain V gene segment, one heavy chain diversity (D) gene segment and one J gene segment, each selected from the appropriate V, D and J families of immunoglobulin gene segments in genomic DNA.

In order to increase the diversity of sequences that contribute to forming antibody binding sites, it is preferable that a heavy chain transgene include cis-acting sequences that support functional V-D-J rearrangement that can incorporate all or part of a D region gene sequence in a rearranged V-D-J gene sequence. Typically, at least about 1 percent of expressed transgene-encoded heavy chains (or mRNAs) include recognizable D region sequences in the V region. Preferably, at least about 10 percent of transgene-encoded V regions include recognizable D region sequences, more preferably at least about 30 percent, and most preferably more than 50 percent include recognizable D region sequences.

A recognizable D region sequence is generally at least about eight consecutive nucleotides corresponding to a sequence present in a D region gene segment of a heavy chain transgene and/or the amino acid sequence encoded by such D region nucleotide sequence. For example, if a transgene includes the D region gene DHQ52, a transgene-encoded mRNA containing the sequence 5'-TAACTGGG-3' located in the V region between a V gene segment sequence and a J gene segment sequence is recognizable as containing a D region sequence, specifically a DHQ52 sequence. Similarly, for example, if a transgene includes the D region gene DHQ52, a transgene-encoded heavy chain polypeptide containing the amino acid sequence —DAF— located in the V region between a V gene segment amino acid sequence and a J gene segment amino acid sequence may be recognizable as containing a D region sequence, specifically a DHQ52 sequence. However, since D region segments may be incorporated in VDJ joining to various extents and in various reading frames, a comparison of the D region area of a heavy chain variable region to the D region segments present in the transgene is necessary to determine the incorporation of particular D segments. Moreover, potential exonuclease digestion during recombination may lead to imprecise V-D and D-J joints during V-D-J recombination.

However, because of somatic mutation and N-region addition, some D region sequences may be recognizable but may not correspond identically to a consecutive D region sequence in the transgene. For example, a nucleotide sequence 5'-CTAAXTGGGG-3' (SEQ ID NO: 1), where X is A, T, or G, and which is located in a heavy chain V region and flanked by a V region gene sequence and a J region gene sequence, can be recognized as corresponding to the DHQ52 sequence 5'-CTAACTGGG-3'. Similarly, for example, the polypeptide sequences —DAFDI— (SEQ ID NO:2), —DYFDY— (SEQ ID NO:3, or —GAFDI— (SEQ ID NO:4) located in a V region and flanked on the amino-terminal side by an amino acid sequence encoded by a transgene V gene sequence and flanked on the carboxyter-minal side by an amino acid sequence encoded by a transgene J gene sequence is recognizable as a D region sequence.

Therefore, because somatic mutation and N-region addition can produce mutations in sequences derived from a transgene D region, the following definition is provided as a guide for determining the presence of a recognizable D region sequence. An amino acid sequence or nucleotide sequence is recognizable as a D region sequence if: (1) the sequence is located in a V region and is flanked on one side by a V gene sequence (nucleotide sequence or deduced amino acid sequence) and on the other side by a J gene sequence (nucleotide sequence or deduced amino acid sequence) and (2) the sequence is substantially identical or substantially similar to a known D gene sequence (nucleotide sequence or encoded amino acid sequence).

The term "substantial identity" as used herein denotes a characteristic of a polypeptide sequence or nucleic acid sequence, wherein the polypeptide sequence has at least 50 percent sequence identity compared to a reference sequence, often at least about 80% sequence identity and sometimes more than about 90% sequence identity, and the nucleic acid sequence has at least 70 percent sequence identity compared to a reference sequence. The percentage of sequence identity is calculated excluding small deletions or additions which total less than 35 percent of the reference sequence. The reference sequence may be a subset of a larger sequence, such as an entire D gene; however, the reference sequence is at least 8 nucleotides long in the case of polynucleotides, and at least 3 amino residues long in the case of a polypeptide. Typically, the reference sequence is at least 8 to 12 nucleotides or at least 3 to 4 amino acids, and preferably the reference sequence is 12 to 15 nucleotides or more, or at least 5 amino acids.

The term "substantial similarity" denotes a characteristic of an polypeptide sequence, wherein the polypeptide sequence has at least 80 percent similarity to a reference sequence. The percentage of sequence similarity is calculated by scoring identical amino acids or positional conservative amino acid substitutions as similar. A positional

the cell surface changes in two ways: the constant region switches to a non-IgM subtype and the sequence of the variable region can be modified by multiple single amino acid substitutions to produce a higher affinity antibody molecule.

As previously indicated, each variable region of a heavy or light Ig chain contains an antigen binding domain. It has been determined by amino acid and nucleic acid sequencing that somatic mutation during the secondary response occurs throughout the V region including the three complementary determining regions (CDR1, CDR2 and CDR3) also referred to as hypervariable regions 1, 2 and 3 (Kabat et al. *Sequences of Proteins of Immunological Interest* (1991) U.S. Department of Health and Human Services, Washington, D.C., incorporated herein by reference. The CDR1 and CDR2 are located within the variable gene segment whereas the CDR3 is largely the result of recombination between V and J gene segments or V, D and J gene segments. Those portions of the variable region which do not consist of CDR1, 2 or 3 are commonly referred to as framework regions designated FR1, FR2, FR3 and FR4. See FIG. **1**. During hypermutation, the rearranged DNA is mutated to give rise to new clones with altered Ig molecules. Those clones with higher affinities for the foreign antigen are selectively expanded by helper T-cells, giving rise to affinity maturation of the expressed antibody. Clonal selection typically results in expression of clones containing new mutation within the CDR1, 2 and/or 3 regions. However, mutations outside these regions also occur which influence the specificity and affinity of the antigen binding domain.

Transgenic Non-human Animals Capable of Producing Heterologous Antibody

Transgenic non-human animals in one aspect of the invention are produced by introducing at least one of the immunoglobulin transgenes of the invention (discussed hereinafter) into a zygote or early embryo of a non-human animal. The non-human animals which are used in the invention generally comprise any mammal which is capable of rearranging immunoglobulin gene segments to produce a primary antibody response. Such nonhuman transgenic animals may include, for example, transgenic pigs, transgenic rats, transgenic rabbits, transgenic cattle, and other transgenic animal species, particularly mammalian species, known in the art. A particularly preferred non-human animal is the mouse or other members of the rodent family.

However, the invention is not limited to the use of mice. Rather, any non-human mammal which is capable of mounting a primary and secondary antibody response may be used. Such animals include non-human primates, such as chimpanzee, bovine, ovine, and porcine species, other members of the rodent family, e.g. rat, as well as rabbit and guinea pig. Particular preferred animals are mouse, rat, rabbit and guinea pig, most preferably mouse.

In one embodiment of the invention, various gene segments from the human genome are used in heavy and light chain transgenes in an unrearranged form. In this embodiment, such transgenes are introduced into mice. The unrearranged gene segments of the light and/or heavy chain transgene have DNA sequences unique to the human species which are distinguishable from the endogenous immunoglobulin gene segments in the mouse genome. They may be readily detected in unrearranged form in the germ line and somatic cells not consisting of B-cells and in rearranged form in B-cells.

In an alternate embodiment of the invention, the transgenes comprise rearranged heavy and/or light immunoglobulin transgenes. Specific segments of such transgenes corresponding to functionally rearranged VDJ or VJ segments, contain immunoglobulin DNA sequences which are also clearly distinguishable from the endogenous immunoglobulin gene segments in the mouse.

Such differences in DNA sequence are also reflected in the amino acid sequence encoded by such human immunoglobulin transgenes as compared to those encoded by mouse B-cells. Thus, human immunoglobulin amino acid sequences may be detected in the transgenic non-human animals of the invention with antibodies specific for immunoglobulin epitopes encoded by human immunoglobulin gene segments.

Transgenic B-cells containing unrearranged transgenes from human or other species functionally recombine the appropriate gene segments to form functionally rearranged light and heavy chain variable regions. It will be readily apparent that the antibody encoded by such rearranged transgenes has a DNA and/or amino acid sequence which is heterologous to that normally encountered in the nonhuman animal used to practice the invention.

Unrearranged Transgenes

As used herein, an "unrearranged immunoglobulin heavy chain transgene" comprises DNA encoding at least one variable gene segment, one diversity gene segment, one joining gene segment and one constant region gene segment. Each of the gene segments of said heavy chain transgene are derived from, or has a sequence corresponding to, DNA encoding immunoglobulin heavy chain gene segments from a species not consisting of the non-human animal into which said transgene is introduced. Similarly, as used herein, an "unrearranged immunoglobulin light chain transgene" comprises DNA encoding at least one variable gene segment, one joining gene segment and at least one constant region gene segment wherein each gene segment of said light chain transgene is derived from, or has a sequence corresponding to, DNA encoding immunoglobulin light chain gene segments from a species not consisting of the non-human animal into which said light chain transgene is introduced.

Such heavy and light chain transgenes in this aspect of the invention contain the above-identified gene segments in an unrearranged form. Thus, interposed between the V, D and J segments in the heavy chain transgene and between the V and J segments on the light chain transgene are appropriate recombination signal sequences (RSS's). In addition, such transgenes also include appropriate RNA splicing signals to join a constant region gene segment with the VJ or VDJ rearranged variable region.

In order to facilitate isotype switching within a heavy chain transgene containing more than one C region gene segment, e.g. Cμ and Cγ1 from the human genome, as explained below "switch regions" are incorporated upstream from each of the constant region gene segments and downstream from the variable region gene segments to permit recombination between such constant regions to allow for immunoglobulin class switching, e.g. from IgM to IgG. Such heavy and light immunoglobulin transgenes also contain transcription control sequences including promoter regions situated upstream from the variable region gene segments which typically contain TATA motifs. A promoter region can be defined approximately as a DNA sequence that, when operably linked to a downstream sequence, can produce transcription of the downstream sequence. Promoters may require the presence of additional linked cis-acting sequences in order to produce efficient transcription. In

addition, other sequences that participate in the transcription of sterile transcripts are preferably included. Examples of sequences that participate in expression of sterile transcripts can be found in the published literature, including Rothman et al., *Intl. Immunol.* 2:621–627 (1990); Reid et al., *Proc. Natl. Acad. Sci. USA* 86:840–844 (1989); Stavnezer et al., *Proc. Natl. Acad. Sci. USA* 85:7704–7708 (1988); and Mills et al., *Nucl. Acids Res.* 18:7305–7316 (1991), each of which is incorporated herein by reference. These sequences typically include about at least 50 bp immediately upstream of a switch region, preferably about at least 200 bp upstream of a switch region; and more preferably about at least 200–1000 bp or more upstream of a switch region. Suitable sequences occur immediately upstream of the human S_{μ}, $S_{\gamma 2}$, $S_{\gamma 3}$, $S_{\gamma 1}$, $S_{\alpha 1}$, $S_{\alpha 2}$, and S_{ε} switch regions; the sequences immediately upstream of the human S_{μ}, and $S_{\gamma 1}$ switch regions can be used to advantage, with $S_{\gamma 1}$ generally preferred. Alternatively, or in combination, murine Ig switch sequences may be used; it may frequently be advantageous to employ Ig switch sequences of the same species as the transgenic non-human animal. Furthermore, interferon (IFN) inducible transcriptional regulatory elements, such as IFN-inducible enhancers, are preferably included immediately upstream of transgene switch sequences.

In addition to promoters, other regulatory sequences which function primarily in B-lineage cells are used. Thus, for example, a light chain enhancer sequence situated preferably between the J and constant region gene segments on the light chain transgene is used to enhance transgene expression, thereby facilitating allelic exclusion. In the case of the heavy chain transgene, regulatory enhancers and also employed. Such regulatory sequences are used to maximize the transcription and translation of the transgene so as to induce allelic exclusion and to provide relatively high levels of transgene expression.

Although the foregoing promoter and enhancer regulatory control sequences have been generically described, such regulatory sequences may be heterologous to the nonhuman animal being derived from the genomic DNA from which the heterologous transgene immunoglobulin gene segments are obtained. Alternately, such regulatory gene segments are derived from the corresponding regulatory sequences in the genome of the non-human animal, or closely related species, which contains the heavy and light transgene.

In the preferred embodiments, gene segments are derived from human beings. The transgenic non-human animals harboring such heavy and light transgenes are capable of mounting an Ig-mediated immune response to a specific antigen administered to such an animal. B-cells are produced within such an animal which are capable of producing heterologous human antibody. After immortalization, and the selection for an appropriate monoclonal antibody (Mab), e.g. a hybridoma, a source of therapeutic human monoclonal antibody is provided. Such human Mabs have significantly reduced immunogenicity when therapeutically administered to humans.

Although the preferred embodiments disclose the construction of heavy and light transgenes containing human gene segments, the invention is not so limited. In this regard, it is to be understood that the teachings described herein may be readily adapted to utilize immunoglobulin gene segments from a species other than human beings. For example, in addition to the therapeutic treatment of humans with the antibodies of the invention, therapeutic antibodies encoded by appropriate gene segments may be utilized to generate monoclonal antibodies for use in the veterinary sciences.

Rearranged Transgenes

In an alternative embodiment, transgenic nonhuman animals contain functionally at least one rearranged heterologous heavy chain immunoglobulin transgene in the germline of the transgenic animal. Such animals contain primary repertoire B-cells that express such rearranged heavy transgenes. Such B-cells preferably are capable of undergoing somatic mutation when contacted with an antigen to form a heterologous antibody having high affinity and specificity for the antigen. Said rearranged transgenes will contain at least two C_H genes and the associated sequences required for isotype switching.

The invention also includes transgenic animals containing germ line cells having heavy and light transgenes wherein one of the said transgenes contains rearranged gene segments with the other containing unrearranged gene segments. In such animals, the heavy chain transgenes shall have at least two C_H genes and the associated sequences required for isotype switching.

The invention further includes methods for generating a synthetic variable region gene segment repertoire to be used in the transgenes of the invention. The method comprises generating a population of immunoglobulin V segment DNAs wherein each of the V segment DNAs encodes an immunoglobulin V segment and contains at each end a cleavage recognition site of a restriction endonuclease. The population of immunoglobulin V segment DNAs is thereafter concatenated to form the synthetic immunoglobulin V segment repertoire. Such synthetic variable region heavy chain transgenes shall have at least two C_H genes and the associated sequences required for isotype switching.

Isotype Switching

In the development of a B lymphocyte, the cell initially produces IgM with a binding specificity determined by the productively rearranged V_H and V_L regions. Subsequently, each B cell and its progeny cells synthesize antibodies with the same L and H chain V regions, but they may switch the isotype of the H chain.

The use of μ or δ constant regions is largely determined by alternate splicing, permitting IgM and IgD to be coexpressed in a single cell. The other heavy chain isotypes (γ, α, and ε) are only expressed natively after a gene rearrangement event deletes the Cμ and Cδ exons. This gene rearrangement process, termed isotype switching, typically occurs by recombination between so called switch segments located immediately upstream of each heavy chain gene (except δ). The individual switch segments are between 2 and 10 kb in length, and consist primarily of short repeated sequences. The exact point of recombination differs for individual class switching events. Investigations which have used solution hybridization kinetics or Southern blotting with cDNA-derived C_H probes have confirmed that switching can be associated with loss of C_H sequences from the cell.

The switch (S) region of the μ gene, S_{μ}, is located about 1 to 2 kb 5′ to the coding sequence and is composed of numerous tandem repeats of sequences of the form $(GAGCT)_n(GGGGT)$ (SEQ ID NOS:9–24), where n is usually 2 to 5 but can range as high as 17. (See T. Nikaido et al. *Nature* 292:845–848 (1981)).

Similar internally repetitive switch sequences spanning several kilobases have been found 5′ of the other C_H genes. The S_{α} region has been sequenced and found to consist of tandemly repeated 80-bp homology units, whereas murine $S_{\gamma 2a}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ all contain repeated 49-bp homology units very similar to each other. (See, P. Szurek et al., *J. Immunol* 135:620–626 (1985) and T. Nikaido et al., *J. Biol. Chem.* 257:7322–7329 (1982), which are incorporated herein by

T is a cis-acting transcriptional regulatory region segment containing at least a promoter,

S_A is an acceptor region segment capable of participating in a recombination event with selected S_D donor region segments, such that isotype switching occurs,

C_Z is a heavy chain constant region genc segment encoding an isotype other than μ (e.g., γ_1, γ_2, γ_3, γ_4, α_1, α_2, ε). x, y, z, m, n, and q are integers. x is 1–100, n is 0–10, y is 1–50, p is 1–10, z is 1–50, q is 0–50, m is 0–10. Typically, when the transgene is capable of isotype switching, q must be at least 1, m is at least 1, n is at least 1, and m is greater than or equal to n.

V_H, D, J_H, S_D, C_1, T, S_A, and C_Z segments may be selected from various species, preferably mammalian species, and more preferably from human and murine germline DNA.

V_H segments may be selected from various species, but are preferably selected from V_H segments that occur naturally in the human germline, such as V_{H251}. Typically about 2 V_H gene segments are included, preferably about 4 V_H segments are included, and most preferably at least about 10 V_H segments are included.

At least one D segment is typically included, although at least 10 D segments are preferably included, and some embodiments include more than ten D segments. Some prefcrred embodiments include human D segments.

Typically at least one J_H segment is incorporated in the transgene, although it is preferable to include about six J_H segments, and some preferred embodiments include more than about six J_H segments. Some preferred embodiments include human J_H segments, and further preferred embodiments include six human J_H segments and no nonhuman J_H segments.

S_D segments are donor regions capable of participating in recombination events with the S_A segment of the transgene. For classical isotype switching, S_D and S_A are switch regions such as S_μ, $S_{\gamma 1}$, $S_{\gamma 2}$, $S_{\gamma 4}$, $S_{\alpha 1}$, $S_{\alpha 2}$, and S_ε. Preferably the S_D switch regions are murine or human, more preferably S_D is a human or murine S_μ and S_A is a human or murine $S_{\gamma 1}$. For nonclassical isotype switching (δ-associated deletion), S_D and S_A are preferably the 400 basepair direct repeat sequences that flank the human μ gene.

C_1 segments are typically μ or δ genes, preferably a μ gene, and more preferably a human or murine μ gene.

T segments typically include 5' flanking sequences that are adjacent to naturally occurring (i.e., germline) switch regions. T segments typically at least about at least 50 nucleotides in length, preferably about at least 200 nucleotides in length, and more preferably at least 500–1000 nucleotides in length. Preferably T segments are 5' flanking sequences that occur immediately upstream of human or murine switch regions in a germline configuration. It is also evident to those of skill in the art that T segments may comprise cis-acting transcriptional regulatory sequences that do not occur naturally in an animal germline (e.g., viral enhancers and promoters such as those found in SV40, adenovirus, and other viruses that infect eukaryotic cells).

C_Z segments are typically a γ_1, γ_2, γ_3, γ_4, α_1, α_2, or ε C_H gene, preferably a human C_H gene of these isotypes, and more preferably a human γ_1 or γ_4 gene. Murine γ_{2a} and γ_{2a} may also be used, as may downstream (i.e., switched) isotype genes form various species. Where the heavy chain transgene contains an immunoglobulin heavy chain minilocus, the total length of the transgene will be typically 150 kilo basepairs or less.

In general, the transgene will be other than a native heavy chain Ig locus. Thus, for example, deletion of unnecessary regions or substitutions with corresponding regions from other species will be present.

F. Methods for Determining Functional Isotype Switching in Ig Transgenes

The occurrence of isotype switching in a transgenic nonhuman animal may be identified by any method known to those in the art. Preferred embodiments include the following, employed either singly or in combination:

1. detection of mRNA transcripts that contain a sequence homologous to at least one transgene downstream C_H gene other than δ and an adjacent sequence homologous to a transgene V_H-D_H-J_H rearranged gene; such detection may be by Northern hybridization, S_1 nuclease protection assays, PCR amplification, cDNA cloning, or other methods;
2. detection in the serum of the transgenic animal, or in supernatants of cultures of hybridoma cells made from B-cells of the transgenic animal, of immunoglobulin proteins encoded by downstream C_H genes, where such proteins can also be shown by immunochemical methods to comprise a functional variable region;
3. detection, in DNA from B-cells of the transgenic animal or in genomic DNA from hybridoma cells, of DNA rearrangements consistent with the occurrence of isotype switching in the transgene, such detection may be accomplished by Southern blot hybridization, PCR amplification, genomic cloning, or other method; or
4. identification of other indicia of isotype switching, such as production of sterile transcripts, production of characteristic enzymes involved in switching (e.g., "switch recombinase"), or other manifestations that may be detected, measured, or observed by contemporary techniques.

Because each transgenic line may represent a different site of integration of the transgene, and a potentially different tandem array of transgene inserts, and because each different configuration of transgene and flanking DNA sequences can affect gene expression, it is preferable to identify and use lines of mice that express high levels of human immunoglobulins, particularly of the IgG isotype, and contain the least number of copies of the transgene. Single copy transgenics minimize the potential problem of incomplete allelic expression. Transgenes are typically integrated into host chromosomal DNA, most usually into germline DNA and propagated by subsequent breeding of germline transgenic breeding stock animals. However, other vectors and transgenic methods known in the present art or subsequently developed may be substituted as appropriate and as desired by a practitioncr.

Trans-switching to endogenous nonhuman heavy chain constant region genes can occur and produce chimeric heavy chains and antibodies comprising such chimeric human/mouse heavy chains. Such chimeric antibodies may be desired for certain uses described herein or may be undesirable.

G. Functional Disruption of Endogenous Immunoglobulin Loci

The expression of successfully rearranged immunoglobulin heavy and light transgenes is expected to have a dominant effect by suppressing the rearrangement of the endogenous immunoglobulin genes in the transgenic nonhuman animal. However, another way to generate a nonhuman that is devoid of endogenous antibodies is by mutating the endogenous immunoglobulin loci. Using embryonic stem cell technology and homologous recombination, the endogenous immunoglobulin repertoire can be readily eliminated. The following describes the functional description of the

mouse immunoglobulin loci. The vectors and methods disclosed, however, can be readily adapted for use in other non-human animals.

Briefly, this technology involves the inactivation of a gene, by homologous recombination, in a pluripotent cell line that is capable of differentiating into germ cell tissue. A DNA construct that contains an altered, copy of a mouse immunoglobulin gene is introduced into the nuclei of embryonic stem cells. In a portion of the cells, the introduced DNA recombines with the endogenous copy of the mouse gene, replacing it with the altered copy. Cells containing the newly engineered genetic lesion are injected into a host mouse embryo, which is reimplanted into a recipient female. Some of these embryos develop into chimeric mice that possess germ cells entirely derived from the mutant cell line. Therefore, by breeding the chimeric mice it is possible to obtain a new line of mice containing the introduced genetic lesion (reviewed by Capecchi (1989), *Science*, 244, 1288–1292).

Because the mouse λ locus contributes to only 5% of the immunoglobulins, inactivation of the heavy chain and/or κ-light chain loci is sufficient. There are three ways to disrupt each of these loci, deletion of the J region, deletion of the J-C intron enhancer, and disruption of constant region coding sequences by the introduction of a stop codon. The last option is the most straightforward, in terms of DNA construct design. Elimination of the μ gene disrupts B-cell maturation thereby preventing class switching to any of the functional heavy chain segments. The strategy for knocking out these loci is outlined below.

To disrupt the mouse μ and κ genes, targeting vectors are used based on the design employed by Jaenisch and co-workers (Zijlstra, et al. (1989), *Nature*, 342, 435–438) for the successful disruption of the mouse β2-microglobulin gene. The neomycin resistance gene (neo), from the plasmid pMClneo is inserted into the coding region of the target gene. The pMClneo insert uses a hybrid viral promoter/enhancer sequence to drive neo expression. This promoter is active in embryonic stem cells. Therefore, neo can be used as a selectable marker for integration of the knock-out construct. The HSV thymidine kinase (tk) gene is added to the end of the construct as a negative selection marker against random insertion events (Zijlstra, et al., supra.).

A preferred strategy for disrupting the heavy chain locus is the elimination of the J region. This region is fairly compact in the mouse, spanning only 1.3 kb. To construct a gene targeting vector, a 15 kb KpnI fragment containing all of the secreted A constant region exons from mouse genomic library is isolated. The 1.3 kb J region is replaced with the 1.1 kb insert from pMClneo. The HSV tk gene is then added to the 5' end of the KpnI fragment. Correct integration of this construct, via homologous recombination, will result in the replacement of the mouse J_H region with the neo gene. Recombinants are screened by PCR, using a primer based on the neo gene and a primer homologous to mouse sequences 5' of the KpnI site in the D region.

Alternatively, the heavy-chain locus is knocked out by disrupting the coding region of the μ gene. This approach involves the same 15 kb KpnI fragment used in the previous approach. The 1.1 kb insert from pMClneo is inserted at a unique BamHI site in exon II, and the HSV tk gene added to the 3' KpnI end. Double crossover events on either side of the neo insert, that eliminate the tk gene, are then selected for. These are detected from pools of selected clones by PCR amplification. One of the PCR primers is derived from neo sequences and the other from mouse sequences outside of the targeting vector. The functional disruption of the mouse immunoglobulin loci is presented in the Examples.

G. Suppressing Expression of Endogenous Immunoglobulin Loci

In addition to functional disruption of endogenous Ig loci, an alternative method for preventing the expression of an endogenous Ig locus is suppression. Suppression of endogenous Ig genes may be accomplished with antisense RNA produced from one or more integrated transgenes, by antisense oligonucleotides, and/or by administration of antisera specific for one or more endogenous Ig chains.

Antisense Polynucleotides

Antisense RNA transgenes can be employed to partially or totally knock-out expression of specific genes (Pepin et al. (1991) *Nature* 355: 725; Helene., C. and Toulme, J. (1990) *Biochimica Biophys. Acta* 1049: 99; Stout, J. and Caskey, T. (1990) *Somat. Cell Mol. Genet.* 16: 369; Munir et al. (1990) *Somat. Cell Mol. Genet.* 16: 383, each of which is incorporated herein by reference).

"Antisense polynucleotides" are polynucleotides that: (1) are complementary to all or part of a reference sequence, such as a sequence of an endogenous Ig C_H or C$_L$ region, and (2) which specifically hybridize to a complementary target sequence, such as a chromosomal gene locus or a Ig mRNA. Such complementary antisense polynucleotides may include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridization to the relevant target sequence is retained as a functional property of the polynucleotide. Complementary antisense polynucleotides include soluble antisense RNA or DNA oligonucleotides which can hybridize specifically to individual mRNA species and prevent transcription and/or RNA processing of the mRNA species and/or translation of the encoded polypeptide (Ching et al., *Proc. Natl. Acad. Sci. U.S.A.* 86:10006–10010 (1989); Broder et al., *Ann. Int. Med.* 113:604–618 (1990); Loreau et al., *FEBS Letters* 274:53–56 - (1990); Holcenberg et al., WO91/11535; U.S. Ser. No. 07/530,165 ("New human CRIPTO gene"); issued as U.S. Pat. No. 5,256,643 on Oct. 26, 1993); WO90/10965; WO91/04753; WO90/13641; and EP 386563, each of which is incorporated herein by reference). An antisense sequence is a polynucleotide sequence that is complementary to at least one immunoglobulin gene sequence of at least about 15 contiguous nucleotides in length, typically at least 20 to 30 nucleotides in length, and preferably more than about 30 nucleotides in length. However, in some embodiments, antisense sequences may have substitutions, additions, or deletions as compared to the complementary immunoglobulin gene sequence, so long as specific hybridization is retained as a property of the antisense polynucleotide. Generally, an antisense sequence is complementary to an endogenous immunoglobulin gene sequence that encodes, or has the potential to encode after DNA rearrangement, an immunoglobulin chain. In some cases, sense sequences corresponding to an immunoglobulin gene sequence may function to suppress expression, particularly by interfering with transcription.

The antisense polynucleotides therefore inhibit production of the encoded polypeptide(s). In this regard, antisense polynucleotides that inhibit transcription and/or translation of one or more endogenous Ig loci can alter the capacity and/or specificity of a non-human animal to produce immunoglobulin chains encoded by endogenous Ig loci.

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or trans-

genic cell, such as a transgenic pluripotent hematopoietic stem cell used to reconstitute all or part of the hematopoietic stem cell population of an individual, or a transgenic non-human animal. Alternatively, the antisense polynucleotides may comprise soluble oligonucleotides that are administered to the external milieu, either in culture medium in vitro or in the circulatory system or interstitial fluid in vivo. Soluble antisense polynucleotides present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species. In some embodiments the antisense polynucleotides comprise methylphosphonate moieties, alternatively phosphorothiolates or O-methylribonucleotides may be used, and chimeric oligonucleotides may also be used (Dagle et al. (1990) *Nucleic Acids Res.* 18: 4751). For some applications, antisense oligonucleotides may comprise polyamide nucleic acids (Nielsen et al. (1991) *Science* 254: 1497). For general methods relating to antisense polynucleotides, see *Antisense RNA and DNA*, (1988), D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y.).

Antisense polynucleotides complementary to one or more sequences are employed to inhibit transcription, RNA processing, and/or translation of the cognate mRNA species and thereby effect a reduction in the amount of the respective encoded polypeptide. Such antisense polynucleotides can provide a therapeutic function by inhibiting the formation of one or more endogenous Ig chains in vivo.

Whether as soluble antisense oligonucleotides or as antisense RNA transcribed from an antisense transgene, the antisense polynucleotides of this invention are selected so as to hybridize preferentially to endogenous Ig sequences at physiological conditions in vivo. Most typically, the selected antisense polynucleotides will not appreciably hybridize to heterologous Ig sequences encoded by a heavy or light chain transgene of the invention (i.e., the antisense oligonucleotides will not inhibit transgene Ig expression by more than about 25 to 35 percent).

Antiserum Suppression

Partial or complete suppression of endogenous Ig chain expression can be produced by injecting mice with antisera against one or more endogenous Ig chains (Weiss et al. (1984) *Proc. Natl. Acad. Sci.* (*U.S.A.*) 81 211, which is incorporated herein by reference). Antisera are selected so as to react specifically with one or more endogenous (e.g., murine) Ig chains but to have minimal or no cross-reactivity with heterologous Ig chains encoded by an Ig transgene of the invention. Thus, administration of selected antisera according to a schedule as typified by that of Weiss et al. *op.cit.* will suppress endogenous Ig chain expression but permits expression of heterologous Ig chain(s) encoded by a transgene of the present invention. Suitable antibody sources for antibody comprise:

(1) monoclonal antibodies, such as a monoclonal antibody that specifically binds to a murine μ, γ, κ, or λ chains but does not react with the human immunoglobulin chain(s) encoded by a human Ig transgene of the invention;

(2) mixtures of such monoclonal antibodies, so that the mixture binds with multiple epitopes on a single species of endogenous Ig chain, with multiple endogenous Ig chains (e.g., murine μ and murine γ, or with multiple epitopes and multiple chains or endogenous immunoglobulins;

(3) polyclonal antiserum or mixtures thereof, typically such antiserum/antisera is monospecific for binding to a single species of endogenous Ig chain (e.g., murine μ, murine γ, murine κ, murine λ) or to multiple species of endogenous Ig chain, and most preferably such antisera possesses negligible binding to human immunoglobulin chains encoded by a transgene of the invention; and/or

(4) a mixture of polyclonal antiserum and monoclonal antibodies binding to a single or multiple species of endogenous Ig chain, and most preferably possessing negligible binding to human immunoglobulin chains encoded by a transgene of the invention. Generally, polyclonal antibodies are preferred, and such substantially monospecific polyclonal antibodies can be advantageously produced from an antiserum raised against human immunoglobulin(s) by pre-adsorption with antibodies derived from the nonhuman animal species (e.g., murine) and/or, for example, by affinity chromatography of the antiserum or purified fraction thereof on an affinity resin containing immobilized human Ig (wherein the bound fraction is enriched for the desired anti-human Ig in the antiserum; the bound fraction is typically eluted with conditions of low pH or a chaotropic salt solution).

Cell separation and/or complement fixation can be employed to provide the enhancement of antibody-directed cell depletion of lymphocytes expressing endogenous (e.g., murine) immunoglobulin chains. In one embodiment, for example, antibodies are employed for ex vivo depletion of murine Ig-exprssing explanted hematopoietic cells and/or B-lineage lymphocytes obtained from a transgenic mouse harboring a human Ig transgene. Thus, hematopoietic cells and/or B-lineage lymphocytes are explanted from a transgenic nonhuman animal harboring a human Ig transgene (preferably harboring both a human heavy chain transgene and a human light chain transgene) and the explanted cells are incubated with an antibody (or antibodies) which (1) binds to an endogenous immunoglobulin (e.g., murine μ and/or κ) and (2) lacks substantial binding to human immunoglobulin chains encoded by the transgene(s). Such antibodies are referred to as "suppression antibodies" for clarity. The explanted cell population is selectively depleted of cells which bind to the suppression antibody(ies);

such depletion can be accomplished by various methods, such as (1) physical separation to remove suppression antibody-bound cells from unbound cells (e.g., the suppression antibodies may be bound to a solid support or magnetic bead to immobilize and remove cells binding to the suppression antibody), (2) antibody-dependent cell killing of cells bound by the suppression antibody (e.g., by ADCC, by complement fixation, or by a toxin linked to the suppression antibody), and (3) clonal anergy induced by the suppression antibody, and the like.

Frequently, antibodies used for antibody suppression of endogenous Ig chain production will be capable of fixing complement. It is frequently preferable that such antibodies may be selected so as to react well with a convenient complement source for ex vivo/in vitro depletion, such as rabbit or guinea pig complement. For in vivo depletion, it is generally preferred that the suppressor antibodies possess effector functions in the nonhuman transgenic animal species;

thus, a suppression antibody comprising murine effector functions (e.g., ADCC and complement fixation) generally would be preferred for use in transgenic mice.

In one variation, a suppression antibody that specifically binds to a predetermined endogenous immunoglobulin chain is used for ex vivo/in vitro depletion of lymphocytes

expressing an endogenous immunoglobulin. A cellular explant (e.g., lymphocyte sample) from a transgenic non-human animal harboring a human immunoglobulin transgene is contacted with a suppression antibody and cells specifically binding to the suppression antibody are depleted (e.g., by immobilization, complement fixation, and the like), thus generating a cell subpopulation depleted in cells expressing endogenous (nonhuman) immunoglobulins (e.g., lymphocytes expressing murine Ig). The resultant depleted lymphocyte population (T cells, human Ig-positive B-cells, etc.) can be transferred into a immunocompatible (i.e., MHC-compatible) nonhuman animal of the same species and which is substantially incapable of producing endogenous antibody (e.g., SCID mice, RAG-1 or RAG-2 knock-out mice). The reconstituted animal (mouse) can then be immunized with an antigen (or reimmunized with an antigen used to immunize the donor animal from which the explant was obtained) to obtain high-affinity (affinity matured) antibodies and B-cells producing such antibodies. Such B-cells may be used to generate hybridomas by conventional cell fusion and screened. Antibody suppression can be used in combination with other endogenous Ig inactivation/suppression methods (e.g., J_H knockout, C_H knockout, D-region ablation, antisense suppression, compensated frameshift inactivation).

Complete Endogenous Ig Locus Inactivation

In certain embodiments, it is desirable to effect complete inactivation of the endogenous Ig loci so that hybrid immunoglobulin chains comprising a human variable region and a non-human (e.g., murine) constant region cannot be formed (e.g., by trans-switching between the transgene and endogenous Ig sequences). Knockout mice bearing endogenous heavy chain alleles with are functionally disrupted in the J_H region only frequently exhibit trans-switching, typically wherein a rearranged human variable region (VDJ) encoded by a transgene is expressed as a fusion protein linked to an endogenous murine constant region, although other trans-switched junctions are possible. To overcome this potential problem, it is generally desirable to completely inactivate the endogenous heavy chain locus by any of various methods, including but not limited to the following: (1) functionally disrupting and/or deleting by homologous recombination at least one and preferably all of the endogenous heavy chain constant region genes, (2) mutating at least one and preferably all of the endogenous heavy chain constant region genes to encode a termination codon (or frameshift) to produce a truncated or frameshifted product (if trans-switched), and other methods and strategies apparent to those of skill in the art. Deletion of a substantial portion or all of the heavy chain constant region genes and/or D-region genes may be accomplished by various methods, including sequential deletion by homologous recombination targeting vectors, especially of the "hit-and-run" type and the like. Similarly, functional disruption and/or deletion of at least one endogenous light chain locus (e.g., κ) to ablate endogenous light chain constant region genes is often preferable.

Frequently, it is desirable to employ a frameshifted transgene wherein the heterologous transgene comprises a frame-shift in the J segment(s) and a compensating frameshift (i.e., to regenerate the original reading frame) in the initial region (i.e., amino-terminal coding portion) of one or more (preferably all) of the transgene constant region genes. Trans-switching to an endogenous IgH locus constant gene (which does not comprise a compensating frameshift) will result in a truncated or missense product that results in the trans-switched B cell being deleted or non-selected, thus suppressing the trans-switched phenotype.

Antisense suppression and antibody suppression may also be used to effect a substantially complete functional inactivation of endogenous Ig gene product expression (e.g., murine heavy and light chain sequences) and/or trans-switched antibodies (e.g., human variable/murine constant chimeric antibodies).

Various combinations of the inactivation and suppression strategies may be used to effect essentially total suppression of endogenous (e.g., murine) Ig chain expression.

Trans-switching

In some variations, it may be desirable to produce a trans-switched immunoglobulin. For example, such trans-switched heavy chains can be chimeric (i.e., a non-murine (human) variable region and a murine constant region). Antibodies comprising such chimeric trans-switched immunoglobulins can be used for a variety of applications where it is desirable to have a non-human (e.g., murine) constant region (e.g., for retention of effector functions in the host, for the presence of murine immunological determinants such as for binding of a secondary antibody which does not bind human constant regions). For one example, a human variable region repertoire may possess advantages as compared to the murine variable region repertoire with respect to certain antigens. Presumably the human V_H, D, J_H, V_L, and J_L genes have been selected for during evolution for their ability to encode immunoglobulins that bind certain evolutionarily important antigens; antigens which provided evolutionary selective pressure for the murine repertoire can be distinct from those antigens which provided evolutionary pressure to shape the human repertoire. Other repertoire advantages may exist, making the human variable region repertoire advantageous when combined with a murine constant region (e.g., a trans-switched murine) isotype. The presence of a murine constant region can afford advantages over a human constant region. For example, a murine γ constant region linked to a human variable region by trans-switching may provide an antibody which possesses murine effector functions (e.g., ADCC, murine complement fixation) so that such a chimeric antibody (preferably monoclonal) which is reactive with a predetermined antigen (e.g., human IL-2 receptor) may be tested in a mouse disease model, such as a mouse model of graft-versus-host disease wherein the T lymphocytes in the mouse express a functional human IL-2 receptor. Subsequently, the human variable region encoding sequence may be isolated (e.g., by PCR amplification or cDNA cloning from the source (hybridoma clone)) and spliced to a sequence encoding a desired human constant region to encode a human sequence antibody more suitable for human therapeutic uses where immunogenicity is preferably minimized. The polynucleotide(s) having the resultant fully human encoding sequence(s) can be expressed in a host cell (e.g., from an expression vector in a mammalian cell) and purified for pharmaceutical formulation. For some applications, the chimeric antibodies may be used directly without replacing the murine constant region with a human constant region. Other variations and uses of trans-switched chimeric antibodies will be evident to those of skill in the art.

The present invention provides transgenic nonhuman animals containing B lymphocytes which express chimeric antibodies, generally resulting from trans-switching between a human heavy chain transgene and an endogenous murine heavy chain constant region gene. Such chimeric

antibodies comprise a human sequence variable region and a murine constant region, generally a murine switched (i.e., non-μ, non-δ) isotype. The transgenic nonhuman animals capable of making chimeric antibodies to a predetermined antigen are usually also competent to make fully human sequence antibodies if both human heavy chain and human light chain transgenes encoding human variable and human constant region genes are integrated. Most typically, the animal is homozygous for a functionally disrupted heavy chain locus and/or light chain locus but retains one or more endogenous heavy chain constant region gene(s) capable of trans-switching (e.g., γ, α, ε) and frequently retains a cis-linked enhancer. Such a mouse is immunized with a predetermined antigen, usually in combination with an adjuvant, and an immune response comprising a detectable amount of chimeric antibodies comprising heavy chains composed of human sequence variable regions linked to murine constant region sequences is produced. Typically, the serum of such an immunized animal can comprise such chimeric antibodies at concentrations of about at least 1 μg/ml, often about at least 10 μg/ml, frequently at least 30 μg/ml, and up to 50 to 100 μg/ml or more. The antiserum containing antibodies comprising chimeric human variable/mouse constant region heavy chains typically also comprises antibodies which comprise human variable/human constant region (complete human sequence) heavy chains. Chimeric trans-switched antibodies usually comprise (1) a chimeric heavy chain composed of a human variable region and a murine constant region (typically a murine gamma) and (2) a human transgene-encoded light chain (typically kappa) or a murine light chain (typically lambda in a kappa knockout background). Such chimeric trans-switched antibodies generally bind to a predetermined antigen (e.g., the immunogen) with an affinity of about at least 1×10^7 M^{-1}, preferably with an affinity of about at least 5×10^7 M^{-1}, more preferably with an affinity of at least 1×10^8 M^{-1} to 1×10^9 M^{-1} or more. Frequently, the predetermined antigen is a human protein, such as for example a human cell surface antigen (e.g., CD4, CD8, IL-2 receptor, EGF receptor, PDGF receptor), other human biological macromolecule (e.g., thrombomodulin, protein C, carbohydrate antigen, sialyl Lewis antigen, L-selectin), or nonhuman disease associated macromolecule (e.g., bacterial LPS, virion capsid protein or envelope glycoprotein) and the like.

The invention provides transgenic nonhuman animals comprising a genome comprising: (1) a homozygous functionally disrupted endogenous heavy chain locus comprising at least one murine constant region gene capable of trans-switching (e.g., in cis linkage to a functional switch recombination sequence and typically to a functional enhancer), (2) a human heavy chain transgene capable of rearranging to encode and express a functional human heavy chain variable region and capable of trans-switching (e.g., having a cis-linked RSS); optionally further comprising (3) a human light chain (e.g., kappa) transgene capable of rearranging to encode a functional human light chain variable region and expressing a human sequence light chain; optionally further comprising (4) a homozygous functionally disrupted endogenous light chain locus (κ, preferably κ and λ); and optionally further comprising (5) a serum comprising an antibody comprising a chimeric heavy chain composed of a human sequence variable region encoded by a human transgene and a murine constant region sequence encoded by an endogenous murine heavy chain constant region gene (e.g., γ1, γ2a, γ2b, γ3).

Such transgenic mice may further comprise a serum comprising chimeric antibodies which bind a predetermined human antigen (e.g., CD4, CD8, CEA) with an affinity of about at least 1×10^8 M^{-1}, preferably with an affinity of about at least 5×10^8 M^{-1}, more preferably with an affinity of at least 1×10^9 M^{-1} to 1×10^{10} M^{-1} or more. Frequently, hybridomas can be made wherein the monoclonal antibodies produced thereby have an affinity of at least 8×10^7 M^{-1}. Chimeric antibodies comprising a heavy chain composed of a murine constant region and a human variable region, often capable of binding to a nonhuman antigen, may also be present in the serum or as an antibody secreted from a hybridoma.

In some variations, it is desirable to generate transgenic mice which have inactivated endogenous mouse heavy chain loci which retain intact heavy chain constant region genes, and which have a human heavy chain transgene capable of trans-switching, and optionally also have a human light chain transgene, optionally with one or more inactivated endogenous mouse light chain loci. Such mice may advantageously produce B cells capable of alternatively expressing antibodies comprising fully human heavy chains and antibodies comprising chimeric (human variable/mouse constant) heavy chains, by trans-switching. The serum of said mice would contain antibodies comprising fully human heavy chains and antibodies comprising chimeric (human variable/mouse constant) heavy chains, preferably in combination with fully human light chains. Hybridomas can be generated from the B cells of said mice.

Generally, such chimeric antibodies can be generated by trans-switching, wherein a human transgene encoding a human variable region (encoded by productive V-D-J rearrangement in vivo) and a human constant region, typically human μ, undergoes switch recombination with a non-transgene immunoglobulin constant gene switch sequence (RSS) thereby operably linking the transgene-encoded human variable region with a heavy chain constant region which is not encoded by said transgene, typically an endogenous murine immunoglobulin heavy chain constant region or a heterologous (e.g., human) heavy chain constant region encoded on a second transgene. Whereas cis-switching refers to isotype-switching by recombination of RSS elements within a transgene, trans-switching involves recombination between a transgene RSS and an RSS element outside the transgene, often on a different chromosome than the chromosome which harbors the transgene.

Trans-switching generally occurs between an RSS of an expressed transgene heavy chain constant region gene and either an RSS of an endogenous murine constant region gene (of a non-μ isotype, typically γ) or an RSS of a human constant region gene contained on a second transgene, often integrated on a separate chromosome.

When trans-switching occurs between an RSS of a first, expressed transgene heavy chain constant region gene (e.g., a μ) and an RSS of a human heavy chain constant region gene contained on a second transgene, a non-chimeric antibody having a substantially fully human sequence is produced. For example and not limitation, a polynucleotide encoding a human heavy chain constant region (e.g., γ1) and an operably linked RSS (e.g., a γ1 RSS) can be introduced (e.g., transfected) into a population of hybridoma cells generated from a transgenic mouse B-cell (or B cell population) expressing an antibody comprising a transgene-encoded human μ chain. The resultant hybridoma cells can be selected for the presence of the introduced polynucleotide and/or for the expression of trans-switched antibody comprising a heavy chain having the variable region (idiotype/antigen reactivity) of the human μ chain and having the constant region encoded by the introduced polynucleotide

sequence (e.g., human γ1). Trans-switch recombination between the RSS of the transgene-encoded human μ chain and the RSS of the introduced polynucleotide encoding a downstream isotype (e.g., γ1) thereby can generate a trans-switched antibody.

The invention also provides a method for producing such chimeric trans-switched antibodies comprising the step of immunizing with a predetermined antigen a transgenic mouse comprising a genome comprising: (1) a homozygous functionally disrupted endogenous heavy chain locus comprising at least one murine constant region gene capable of trans-switching (e.g., γ2a, γ2b, γ1, γ3), (2) a human heavy chain transgene capable of rearranging to encode a functional human heavy chain variable region and expressing a human sequence heavy chain and capable of undergoing isotype switching (and/or trans-switching), and optionally further comprising (3) a human light chain (e.g., kappa) transgene capable of rearranging to encode a functional human light (e.g., kappa) chain variable region and expressing a human sequence light chain, and optionally further comprising (4) a homozygous functionally disrupted endogenous light chain locus (typically κ, preferably both κ and λ), and optionally further comprising (5) a serum comprising an antibody comprising a chimeric heavy chain composed of a human sequence variable region encoded by a human transgene and a murine constant region sequence encoded by an endogenous murine heavy chain constant region gene (e.g., γ1, γ2a, γ2b, γ3).

Affinity Tagging: Selecting for Switched Isotypes

Advantageously, trans-switching (and cis-switching) is associated with the process of somatic mutation. Somatic mutation expands the range of antibody affinities encoded by clonal progeny of a B-cell. For example, antibodies produced by hybridoma cells which have undergone switching (trans- or cis-) represent a broader range of antigen-binding affinities than is present in hybridoma cells which have not undergone switching. Thus, a hybridoma cell population (typically clonal) which expresses a first antibody comprising a heavy chain comprising a first human heavy chain variable region in polypeptide linkage to a first human heavy chain constant region (e.g., μ) can be screened for hybridoma cell clonal variants which express an antibody comprising a heavy chain containing said first human heavy chain variable region in polypeptide linkage to a second heavy chain constant region (e.g., a human γ, α, or ε constant region). Such clonal variants can be produced by natural clonal variation producing cis-switching in vitro, by induction of class switching (trans- or cis-) as through the administration of agents that promote isotype switching, such as T-cell-derived lymphokines (e.g., IL-4 and IFN$_\gamma$), by introduction of a polynucleotide comprising a functional RSS and a heterologous (e.g. human) heavy chain constant region gene to serve as a substrate for trans-switching, or by a combination of the above, and the like. Often, polynucleotides containing a human downstream isotype constant region (e.g., γ1, γ3, and the like) with an operably linked RSS will also be introduced into hybridoma cells to promote isotype switching via the trans-switch mechanism.

Class switching and affinity maturation take place within the same population of B cells derived from transgenic animals of the present invention. Therefore, identification of class-switched B cells (or hybridomas derived therefrom) can be used as a screening step for obtaining high affinity monoclonal antibodies. A variety of approaches can be employed to facilitate class switching events such as cis-switching (intratransgene switching), trans-switching, or both. For example, a single continuous human genomic fragment comprising both μ and γ constant region genes with the associated RSS elements and switch regulatory elements (e.g., sterile transcript promoter) can be used as a transgene. However, some portions of the desired single contiguous human genomic fragment can be difficult to clone efficiently, such as due to instability problems when replicated in a cloning host or the like; in particular, the region between δ and γ3 can prove difficult to clone efficiently, especially as a contiguous fragment comprising the μ gene, γ3 gene, a V gene, D gene segments, and J gene segments.

Also for example, a discontinuous human transgene (minigene) composed of a human μ gene, human γ3 gene, a human V gene(s), human D gene segments, and human J gene segments, with one or more deletions of an intervening (intronic) or otherwise nonessential sequence (e.g., one or more V, D, and/or J segment and/or one or more non-μ constant region gene(s)). Such minigenes have several advantages as compared to isolating a single contiguous segment of genomic DNA spanning all of the essential elements for efficient immunoglobulin expression and switching. For example, such a minigene avoids the necessity of isolating large pieces of DNA which may contain sequences which are difficult to clone (e.g., unstable sequences, poison sequences, and the like). Moreover, miniloci comprising elements necessary for isotype switching (e.g., human γ sterile transcript promoter) for producing cis- or trans-switching, can advantageously undergo somatic mutation and class switching in vivo. As many eukaryotic DNA sequences can prove difficult to clone, omitting non-essential sequences can prove advantageous.

In a variation, hybridoma clones producing antibodies having high binding affinity (e.g., at least 1×10^7 M^{-1}, preferably at least 1×10^8 M^{-1}, more preferably at least 1×10^9 M^{-1} or greater) are obtained by selecting, from a pool of hybridoma cells derived from B cells of transgenic mice harboring a human heavy chain transgene capable of isotype switching (see, supra) and substantially lacking endogenous murine heavy chain loci capable of undergoing productive (in-frame) V-D-J rearrangement, hybridomas which express an antibody comprising a heavy chain comprising a human sequence heavy chain variable region in polypeptide linkage to a human (or mouse) non-μ heavy chain constant region; said antibodys are termed "switched antibodies" as they comprise a "switched heavy chain" which is produced as a consequence of cis-switching and/or trans-switching in vivo or in cell culture. Hybridomas producing switched antibodies generally have undergone the process of somatic mutation, and a pool of said hybridomas will generally have a broader range of antigen binding affinities from which hybridoma clones secreting high affinity antibodies can be selected. Typically, hybridomas secreting a human sequence antibody having substantial binding affinity (greater than 1×10^7 M^{-1} to 1×10^8 M^{-1}) for a predetermined antigen and wherein said human sequence antibody comprises human immunoglobulin variable region(s) can be selected by a method comprising a two-step process. One step is to identify and isolate hybridoma cells which secrete immunoglobulins which comprise a switched heavy chain (e.g., by binding hybridoma cells to an immobilized immunoglobulin which specifically binds a switched heavy chain and does not substantially bind to an unswitched isotype, e.g., μ). The other step is to identify hybridoma cells which bind to the predetermined antigen with substantial binding affinity (e.g., by ELISA of hybridoma clone supernatants, FACS analysis using labeled antigen, and the like). Typically, selection of hybridomas which secrete switched antibodies

is performed prior to identifying hybridoma cells which bind predetermined antigen. Hybridoma cells which express switched antibodies that have substantial binding affinity for the predetermined antigen are isolated and cultured under suitable growth conditions known in the art, typically as individual selected clones. Optionally, the method comprises the step of culturing said selected clones under conditions suitable for expression of monoclonal antibodies; said monoclonal antibodies are collected and can be administered for therapeutic, prophylactic, and/or diagnostic purposes.

Often, the selected hybridoma clones can serve as a source of DNA or RNA for isolating immunoglobulin sequences which encode immunoglobulins (e.g., a variable region) that bind to (or confer binding to) the predetermined antigen. Subsequently, the human variable region encoding sequence may be isolated (e.g., by PCR amplification or cDNA cloning from the source (hybridoma clone)) and spliced to a sequence encoding a desired human constant region to encode a human sequence antibody more suitable for human therapeutic uses where immunogenicity is preferably minimized. The polynucleotide(s) having the resultant fully human encoding sequence(s) can be expressed in a host cell (e.g., from an expression vector in a mammalian cell) and purified for pharmaceutical formulation.

Xenocnhancers

A heterologous transgene capable of encoding a human immunoglobulin (e.g., a heavy chain) advantageously comprises a cis-linked enhancer which is not derived from the mouse genome, and/or which is not naturally associated in cis with the exons of the heterologous transgene. For example, a human K transgene (e.g., a κ minilocus) can advantageously comprise a human Vκ gene, a human Jκ gene, a human Cκ gene, and a xenoenhancer, typically said xenoenhancer comprises a human heavy chain intronic enhancer and/or a murine heavy chain intronic enhancer, typically located between a Jκ gene and the Cκ gene, or located downstream of the Cκ gene. For example, the mouse heavy chain J-μ intronic enhancer (Banerji et al. (1983) *Cell* 33: 729) can be isolated on a 0.9 kb XbaI fragment of the plasmid pKVe2 (see, infra). The human heavy chain J-μ intronic enhancer (Hayday et al. (1984) *Nature* 307: 334) can be isolated as a 1.4 kb MluI/HindIII fragment (see, infra). Addition of a transcriptionally active xenoenhancer to a transgene, such as a combined xenoenhancer consisting essentially of a human J-μ intronic enhancer linked in cis to a mouse J-μ intronic enhancer, can confer high levels of expression of the transgene, especially where said transgene encodes a light chain, such as human κ. Similarly, a rat 3' enhancer can be advantageously included in a minilocus construct capable of encoding a human heavy chain.

Specific Preferred Embodiments

A preferred embodiment of the invention is an animal containing at least one, typically 2–10, and sometimes 25–50 or more copies of the transgene described in Example 12 (e.g., pHC1 or pHC2) bred with an animal containing a single copy of a light chain transgene described in Examples 5, 6, 8, or 14, and the offspring bred with the J_H deleted animal described in Example 10. Animals are bred to homozygosity for each of these three traits. Such animals have the following genotype: a single copy (per haploid set of chromosomes) of a human heavy chain rearranged mini-locus (described in Example 12), a single copy (per haploid set of chromosomes) of a rearranged human κ light chain construct (described in Example 14), and a deletion at each endogenous mouse heavy chain locus that removes all of the functional J_H segments (described in Example 10). Such animals are bred with mice that are homozygous for the deletion of the J_H segments (Examples 10) to produce offspring that are homozygous for the J_H deletion and hemizygous for the human heavy and light chain constructs. The resultant animals are injected with antigens and used for production of human monoclonal antibodies against these antigens.

B cells isolated from such an animal are monospecific with regard to the human heavy and light chains because they contain only a single copy of each gene. Furthermore, they will be monospecific with regards to human or mouse heavy chains because both endogenous mouse heavy chain gene copies are nonfunctional by virtue of the deletion spanning the J_H region introduced as described in Example 9 and 12. Furthermore, a substantial fraction of the B cells will be monospecific with regards to the human or mouse light chains because expression of the single copy of the rearranged human κ light chain gene will allelically and isotypically exclude the rearrangement of the endogenous mouse κ and λ chain genes in a significant fraction of B-cells.

The transgenic mouse of the preferred embodiment will exhibit immunoglobulin production with a significant repertoire, ideally substantially similar to that of a native mouse. Thus, for example, in embodiments where the endogenous Ig genes have been inactivated, the total immunoglobulin levels will range from about 0.1 to 10 mg/ml of serum, preferably 0.5 to 5 mg/ml, ideally at least about 1.0 mg/ml. When a transgene capable of effecting a switch to IgG from IgM has been introduced into the transgenic mouse, the adult mouse ratio of serum IgG to IgM is preferably about 10:1. Of course, the IgG to IgM ratio will be much lower in the immature mouse. In general, greater than about 10%, preferably 40 to 80% of the spleen and lymph node B cells express exclusively human IgG protein.

The repertoire will ideally approximate that shown in a non-transgenic mouse, usually at least about 10% as high, preferably 25 to 50% or more. Generally, at least about a thousand different immunoglobulins (ideally IgG), preferably 10^4 to 10^6 or more, will be produced, depending primarily on the number of different V, J and D regions introduced into the mouse genome. These immunoglobulins will typically recognize about one-half or more of highly antigenic proteins, including, but not limited to: pigeon cytochrome C, chicken lysozyme, pokeweed mitogen, bovine serum albumin, keyhole limpit hemocyanin, influenza hemagglutinin, staphylococcus protein A, sperm whale myoglobin, influenza neuraminidase, and lambda repressor protein. Some of the immunoglobulins will exhibit an affinity for preselected antigens of at least about $10^7 M^{-1}$, preferably $10^8 M^{-1}$ to $10^9 M^{-1}$ or greater.

In some embodiments, it may be preferable to generate mice with predetermined repertoires to limit the selection of V genes represented in the antibody response to a predetermined antigen type. A heavy chain transgene having a predetermined repertoire may comprise, for example, human V_H genes which are preferentially used in antibody responses to the predetermined antigen type in humans. Alternatively, some V_H genes may be excluded from a defined repertoire for various reasons (e.g., have a low likelihood of encoding high affinity V regions for the predetermined antigen; have a low propensity to undergo somatic mutation and affinity sharpening; or are immunogenic to certain humans).

Thus, prior to rearrangement of a transgene containing various heavy or light chain gene segments, such gene segments may be readily identified, e.g., by hybridization or DNA sequencing, as being from a species of organism other than the transgenic animal.

The transgenic mice of the present invention can be immunized with a predetermined antigen, such as a transmembrane proteins, cell surface macromolecule, or other suitable antigen (e.g., TNF, LPS, etc.) for which a human antibody would be desirable. The mice will produce B cells which undergo class-switching via intratransgene switch recombination (cis-switching) and express immunoglobulins reactive with the predetermined antigen. The immunoglobulins can be human sequence antibodies, wherein the heavy and light chain polypeptides are encoded by human transgene sequences, which may include sequences derived by somatic mutation and V region recombinatorial joints, as well as germline-encoded sequences; these human sequence immunoglobulins can be referred to as being substantially identical to a polypeptide sequence encoded by a human V_L or V_H gene segment and a human J_L or J_L segment, even though other non-germline sequences may be present as a result of somatic mutation and differential V-J and V-D-J recombination joints. With respect to such human sequence antibodies, the variable regions of each chain are typically at least 80 percent encoded by human germline V, J, and, in the case of heavy chains, D, gene segments; frequently at least 85 percent of the variable regions are encoded by human germline sequences present on the transgene; often 90 or 95 percent or more of the variable region sequences are encoded by human germline sequences present on the transgene. However, since non-germline sequences are introduced by somatic mutation and VJ and VDJ joining, the human sequence antibodies will frequently have some variable region sequences (and less frequently constant region sequences) which are not encoded by human V, D, or J gene segments as found in the human transgene(s) in the germline of the mice. Typically, such non-germline sequences (or individual nucleotide positions) will cluster in or near CDRS, or in regions where somatic mutations are known to cluster.

The human sequence antibodies which bind to the predetermined antigen can result from isotype switching, such that human antibodies comprising a human sequence γ chain (such as γ1, γ2a, γ2B, or γ3) and a human sequence light chain (such as K) are produced. Such isotype-switched human sequence antibodies often contain one or more somatic mutation(s), typically in the variable region and often in or within about 10 residues of a CDR) as a result of affinity maturation and selection of B cells by antigen, particularly subsequent to secondary (or subsequent) antigen challenge. These high affinity human sequence antibodies may have binding affinities of at least 1×10^9 M^{-1}, typically at least $5\times10^9 M^{-1}$, frequently more than 1×10^{10} M^{-1}, and sometimes $5\times10^{10} M^{-1}$ to 1×10^{11} or greater. Such high affinity human sequence antibodies can be made with high binding affinities for human antigens, such as human CD4 and the like human macromolecules (e.g., such as a human transmembrane or cell surface protein or other cell surface antigen).

The B cells from such mice can be used to generate hybridomas expressing monoclonal high affinity (greater than 2×10^9 M^{-1}) human sequence antibodies against a variety of antigens, including human proteins such as CD4 and the like. These hybridomas can be used to generate a composition comprising an immunoglobulin having an affinity constant (K_a) of at least 2×10^9 M^{-1} for binding to a predetermined human antigen, wherein said immunoglobulin consists of:

a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a heavy chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, optionally a D region, and a human J_H segment, and (2) a constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_H gene segment.

Often, the human sequence heavy chain and human sequence light chain are separately encoded by a human heavy chain transgene and a human light chain transgene, respectively, which are integrated into a mouse cell genome. However, both chains may be encoded on a single transgene, or one or both chains may be encoded on multiple transgenes, such as a human heavy chain transgene (e.g., HC2) which derived a V gene segment from a YAC containing a V_H array which is not integrated are the same locus as the human heavy chain transgene in the mouse germline.

In one embodiment, the composition has an immunoglobulin which comprises a human sequence light chain having a κ constant region and a human sequence heavy chain having a γ constant region.

The mice (and hybridomas derived therefrom) are a source for an immunoglobulin having an affinity constant (K_a) of at least 1×10^{10} M^{-1} for binding to a predetermined human antigen, wherein said immunoglobulin consists of:

a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2) a light chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a heavy chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, optionally a D region, and a human J_H segment, and (2) a constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_H gene segment.

The invention provides a transgenic mouse comprising: a homozygous pair of functionally disrupted endogenous heavy chain alleles, a homozygous pair of functionally disrupted endogenous light chain alleles, at least one copy of a heterologous immunoglobulin light chain transgene, and at least one copy of a heterologous immunoglobulin heavy chain transgene, and wherein said animal makes an antibody response following immunization with a human antigen wherein the antibody response comprises an immunoglobulin having an affinity constant (K_a) of at least 2×10^9 M^{-1} for binding to a predetermined human antigen, wherein said immunoglobulin consists of:

a human sequence light chain composed of (1) a light chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_L gene segment and a human J_L segment, and (2)

a light chain constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_L gene segment; and

a human sequence heavy chain composed of a (1) a heavy chain variable region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human V_H gene segment, optionally a D region, and a human J_H segment, and (2) a constant region having a polypeptide sequence which is substantially identical to a polypeptide sequence encoded by a human C_H gene segment.

Such a transgenic mouse can produce a human sequence immunoglobulin which binds to a human surface or transmembrane protein present on at least one somatic cell type of a human, wherein the immunoglobulin binds said human surface or transmembrane protein with an affinity constant (K_a) of between 1.5×10^9 M^{-1} and 1.8×10^{10} M^{-1}. One example of such a human surface or transmembrane protein is CD4, although others may be used as immunogens as desired.

The development of high affinity human sequence antibodies against predetermined antigens is facilitated by a method for expanding the repertoire of human variable region gene segments in a transgenic mouse having a genome comprising an integrated human immunoglobulin transgene, said method comprising introducing into the genome a V gene transgene comprising V region gene segments which are not present in said integrated human immunoglobulin transgene. Often, the V region transgene is a yeast artificial chromosome comprising a portion of a human V_H or V_L (V_K) gene segment array, as may naturally occur in a human genome or as may be spliced together separately by recombinant methods, which may include out-of-order or omitted V gene segments. Often at least five or more functional V gene segments are contained on the YAC. In this variation, it is possible to make a transgenic mouse produced by the V repertoire expansion method, wherein the mouse expresses an immunoglobulin chain comprising a variable region sequence encoded by a V region gene segment present on the V region transgene and a C region encoded on the human Ig transgene. By means of the V repertoire expansion method, transgenic mice having at least 5 distinct V genes can be generated; as can mice containing at least about 24 V genes or more. Of course, some V gene segments may be non-functional (e.g., pseudogenes and the like); these segments may be retained or may be selectively deleted by recombinant methods available to the skilled artisan, if desired.

Once the mouse germline has been engineered to contain a functional YAC having an expanded V segment repertoire, substantially not present in the human Ig transgene containing the J and C gene segments, the trait can be propagated and bred into other genetic backgrounds, including backgrounds where the functional YAC having an expanded V segment repertoire is bred into a mouse germline having a different human Ig transgene. Multiple functional YACs having an expanded V segment repertoire may be bred into a germline to work with a human Ig transgene (or multiple human Ig transgenes). Although referred to herein as YAC transgenes, such transgenes when integrated into the genome may substantially lack yeast sequences, such as sequences required for autonomous replication in yeast; such sequences may optionally be removed by genetic engineering (e.g., restriction digestion and pulsed-field gel electrophoresis or other suitable method) after replication in yeast in no longer necessary (i.e., prior to introduction into a mouse ES cell or mouse prozygote).

The invention also provides a method of propagating the trait of human sequence immunoglobulin expression, comprising breeding a transgenic mouse having the human Ig transgene(s), and optionally also having a functional YAC having an expanded V segment repertoire. Both V_H and V_L gene segments may be present on the YAC. The transgenic mouse may be bred into any background desired by the practitioner, including backgrounds harboring other human transgenes, including human Ig transgenes and/or transgenes encoding other human lymphocyte proteins.

The invention also provides a high affinity human sequence immunoglobulin produced by a transgenic mouse having an expanded V region repertoire YAC transgene.

Although the foregoing describes a preferred embodiment of the transgenic animal of the invention, other embodiments are defined by the disclosure herein and more particularly by the transgenes described in the Examples. Four categories of transgenic animal may be defined:

I. Transgenic animals containing an unrearranged heavy and rearranged light immunoglobulin transgene.

II. Transgenic animals containing an unrearranged heavy and unrearranged light immunoglobulin transgene

III. Transgenic animal containing rearranged heavy and an unrearranged light immunoglobulin transgene, and

IV. Transgenic animals containing rearranged heavy and rearranged light immunoglobulin transgenes.

Of these categories of transgenic animal, the preferred order of preference is as follows II>I>III>IV where the endogenous light chain genes (or at least the κ gene) have been knocked out by homologous recombination (or other method) and I>II>III>IV where the endogenous light chain genes have not been knocked out and must be dominated by allelic exclusion.

As is discussed supra, the invention provides human sequence monoclonal antibodies that are useful in treatment of human diseases. Therapeutic uses of monoclonal antibodies are discussed in, e.g., Larrick and Bourla, *Journal of Biological Response Modifiers*, 5:379–393, which is incorporated herein by reference. Uses of human monoclonal antibodies include treatment of autoimmune diseases, cancer, infectious diseases, transplant rejection, blood disorders such as coagulation disorders, and other diseases.

The antibodies of this invention may be administered to patients by any method known in the medical arts for delivery of proteins. Antibodies are particularly suited for parenteral administration (i.e, subcutaneous, intramuscular or intravenous administration). The pharmaceutical compositions of the present invention are suitable for administration using alternative drug delivery approaches as well (see, e.g., Langer, *Science*, 249:1527–1533 (1990)).

Pharmaceutical compositions for parenteral administration usually comprise a solution of a monoclonal antibody dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH-adjusting and buffering agents, tonicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 0.1% to as much as 1.5% or 2.0% by weight and will be selected primarily based on

fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Sciences, 17th Ed., Mack Publishing Company, Easton, Pa. (1985), which is incorporated herein by reference.

The compositions containing the present antibodies or a cocktail thereof can be administered for the prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient in an amount sufficient to cure or at least partially arrest the infection and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use generally range from about 0.05 mg/kg body weight to about 5 mg/kg body weight, preferably between about 0.2 mg/kg body weight to about 1.5 mg/kg body weight.

In some instances it will be desirable to modify the immunoglobulin molecules of the invention to change their biological activity. For example, the immunoglobulins can be directly or indirectly coupled to other chemotherapeutics agent. A variety of chemotherapeutics can be coupled for targeting. For example, anti-inflammatory agents which may be coupled include immunomodulators, platelet activating factor (PAF) antagonists, cyclooxygenase inhibitors, lipoxygenase inhibitors, and leukotriene antagonists. Some preferred moieties include cyclosporin A, indomethacin, naproxen, FK-506, mycophenolic acid, and the like. Similarly, anti-oxidants, e.g., superoxide dismutase, are useful in treating reperfusion injury. Likewise, anticancer agents, such as daunomycin, doxorubicin, vinblastine, bleomycin, and the like can be targeted.

The monoclonal antibodies of the invention may also be used to target amphipaths (e.g., liposomes) to sites in a patient. In these preparations, the drug to be delivered is incorporated as part of a liposome in which a human monoclonal antibody is embedded.

The human-sequence monoclonal antibodies of the invention are useful, in part, because they bind specifically to the predetermined antigen against which they are directed. When the predetermined antigen is a human antigen (i.e., a human protein or fragment thereof), it will sometimes be advantageous if the human immunoglobulin of the invention also binds to the cognate antigen found in non-human animals, especially animals that are used frequently for drug testing (e.g., preclinical testing of biological activity, pharmacokinetics and safety). These animals include mice, rabbits, rats, dogs, pigs, and, especially, non-human primates such as chimpanzees, apes and monkeys (e.g., Rhesus monkeys and cynomolgus monkeys). The ability to recognize antigens in experimental animals is particularly useful for determining the effect of specific binding on biodistribution of the immunoglobulins. A cognate antigen is an antigen that (i) has a structure (e.g., amino acid sequence) that is substantially similar to the human antigen (i.e., the amino acid sequence of an animal cognate protein will typically be at least about 50% identical to the human protein, usually at least about 70% identical and often at least about 80% identical or more); (ii) has substantially the same function as the human antigen; and, (iii) often is found in the same cellular compartment as the human antigen. Human and animal cognate antigens typically (but not always) have the same names. Examples of cognate antigens include human tubulin and mouse tubulin, human CD4 and Rhesus CD4, and human IgG and Rat IgG.

An other aspect, the invention provides antigen-binding human mABs comprising at least one polypeptide encoded by an artificial gene. An artificial gene comprises a polypeptide-encoding nucleic acid segment that is synthesized in vitro by chemical or enzymatic methods that do not require a cell-derived template nucleic acid strand (e.g., a nucleic acid template obtained from a bacterial cell or an immune or hybridoma cell) and the progeny (through replication) of the artificial gene, i.e., a wholly synthetic nucleic acid.

Although it is routine in genetic engineering to use short synthetic nucleic acids as primers, linkers and the like, it is also possible by chemical and/or enzymatic means to produce wholly synthetic protein-coding nucleic acids that are 30, 50, or more bases in length. The artificial genes of the invention may include both synthetic nucleic acid regions and cell-derived nucleic acid regions. The synthetic nucleic acid region of the artificial gene will generally be at least about 50 bases in length, often at least about 100 bases, typically at least about 200 bases, more often at least about 250 bases and usually over 300 bases or 400 bases in length. Typically the synthetic nucleic acid regions will encode variable gene segments or a portion thereof, e.g., CDR regions, and the constant regions will be encoded by cell-derived nucleic acids. Immunoglobulin polypeptides (i.e., immunoglobulin heavy chains and immunoglobulin light chains) can be conveniently expressed using artificial genes that encode the polypeptides. Usually the artificial genes are operably linked to transcription promoter sequences, e.g., promoter sequences derived from immunoglobulin genes or from viruses (e.g., SV40, CMV, HIV, RSV) or hybrid promoters. The artificial gene may be linked to other sequences as well, [e.g.,] polyadenylation sequences and introns. One method for expressing an immunoglobulin polypeptide involves insertion of a synthetic nucleic acid encoding one region of an immunoglobulin polypeptide (e.g., a variable region or portion thereof) into a vector that encodes the remaining segments or parts of the immunoglobulin chain (e.g., a μ, γ, $\gamma 2$, $\gamma 3$, $\gamma 4$, δ, ϵ, α_1 or α_2 constant region) and, optionally, promoter (e.g., a CMV (cytomegalovirus) promoter), polyadenylation or other sequences. Such vectors are constructed so that upon induction into a cell, the cellular transcription and translation of the vector sequences results in an immunoglobin polypeptide.

Functional human sequence immunoglobulin heavy and light chain genes and polypeptides can be constructed using artificial genes, and used to produce immunoglobulins with a desired specificity such as specific binding to a predetermined antigen. This is accomplished by constructing an artificial gene that encodes an immunoglobulin polypeptide substantially similar to a polypeptide expressed by a cell from, or a hybridoma derived from, a transgenic animal immunized with the predetermined antigen. Thus, the invention provides artificial genes encoding immunoglobulin polypeptides and methods for producing a human-sequence immunoglobulin using an artificial gene(s).

According to this method, a transgenic animal (e.g., a transgenic mouse with a homozygous pair of functionally disrupted endogenous heavy chain alleles, a homozygous pair of functionally disrupted endogenous light chain alleles, at least one copy of a human immunoglobulin light chain transgene, and at least one copy of a human immunoglobulin heavy chain transgene) is immunized with predetermined antigen, e.g., a human protein. Nucleic acid, preferably mRNA, is then collected or isolated from a cell or population of cells in which immunoglobulin gene rearrangement

has taken place, and the sequence(s) of nucleic acids encoding the heavy and/or light chains (especially the V segments) of immunoglobulins, or a portion thereof, is determined. This sequence information is used as a basis for the sequence of the artificial gene.

Sequence determination will generally require isolation of at least a portion of the gene or cDNA of interest, e.g., a portion of a rearranged human transgene or corresponding cDNA encoding an immunoglobulin polypeptide. Usually this requires cloning the DNA or, preferably, mRNA (i.e., cDNA) encoding the human immunoglobulin polypeptide. Cloning is carried out using standard techniques (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Guide*, Vols 1–3, Cold Spring Harbor Press, which is incorporated herein by reference). For example, a cDNA library may be constructed by reverse transcription of polyA+ mRNA, preferably membrane-associated mRNA, and the library screened using probes specific for human immunoglobulin polypeptide gene sequences. In a preferred embodiment, however, the polymerase chain reaction (PCR) is used to amplify cDNAs (or portions of full-lenght cDNAs) encoding an immunoglobulin gene segment of interest (e.g., a light chain variable segment). Because the sequences of the human immunoglobulin polypeptide genes are readily available to those of skill, probes or PCR primers that will specifically hybridize to or amplify a human immunoglobulin gene or segment thereof can be easily designed. See, e.g., Taylor et al., *Nuc. Acids. Res.*, 20:6287 (1992) which is incorporated by reference. Moreover, the sequences of the human transgene of the transgenic mouse will often be known to the practiconer, and primer sequences can be chosen that hybridize to appropriate regions of the transgene. The amplified sequences can be readily cloned into any suitable vector, e.g., expression vectors, minigene vectors, or phage display vectors. It will be appreciated that the particular method of cloning used not critical, so long as it is possible to determine the sequence of some portion of the immunoglobulin polypeptide of interest. As used herein, a nucleic acid that is cloned, amplified, tagged, or otherwise distinguished from background nucleic acids such that the sequence of the nucleic acid of interest can be determined, is considered isolated.

One source for RNA used for cloning and sequencing is a hybridoma produced by obtaining a B cell from the transgenic mouse and fusing the B cell to an immortal cell. An advantage of using hybridomas is that they can be easily screened, and a hybridoma that produces a human monoclonal antibody of interest selected. Alternatively, RNA can be isolated from B cells (or whole spleen) of the immunized animal. When sources other than hybridomas are used, it may be desirable to screen for sequences encoding immunoglobulins or immunoglobulin polypeptides with specific binding characteristics. One method for such screening is the use of phage display technology. Phage display is described in e.g., Dower et al., WO 91/17271, McCafferty et al., WO 92/01047, and Caton and Koprowski, *Proc. Natl. Acad. Sci. USA*, 87:6450–6454 (1990), each of which is incorporated herein by reference. In one embodiment using phage display technology, cDNA from an immunized transgenic mouse (e.g., total spleen cDNA) is isolated, the polymerase chain reaction is used to amplify a cDNA sequences that encode a portion of an immunoglobulin polypeptide, e.g., CDR regions, and the amplified sequences are inserted into a phage vector. cDNAs encoding peptides of interest, e.g., variable region peptides with desired binding characteristics, are identified by standard techniques such as panning.

The sequence of the amplified or cloned nucleic acid is then determined. Typically the sequence encoding an entire variable region of the immunoglobulin polypeptide is determined, however, it will sometimes by adequate to sequence only a portion of a variable region, for example, the CDR-encoding portion. Typically the portion sequenced will be at least 30 bases in length, more often based coding for at least about one-third or at least about one-half of the length of the variable region will be sequenced.

Sequencing can be carried on clones isolated from a cDNA library, or, when PCR is used, after subcloning the amplified sequence or by direct PCR sequencing of the amplified segment. Sequencing is carried out using standard techniques (see, e.g., Sambrook et al. (1989) *Molecular Cloning: A Laboratory Guide*, Vols 1–3, Cold Spring Harbor Press, and Sanger, F. et al. (1977) *Proc. Natl. Acad. Sci. USA* 74: 5463–5467, which is incorporated herein by reference). By comparing the sequence of the cloned nucleic acid with published sequences of human immunoglobulin genes and cDNAs, one of skill will readily be able to determine, depending on the region sequenced, (i) the germline segment usage of the hybridoma immunoglobulin polypeptide (including the isotype of the heavy chain) and (ii) the sequence of the heavy and light chain variable regions, including sequences resulting from N-region addition and the process of somatic mutation. One source of immunoglobulin gene sequence information is the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.

In an alternative embodiment, the amino acid sequence of an immunoglobulin of interest may be determined by direct protein sequencing.

An artificial gene can be constructed that has a sequence identical to or substantially similar to, at least a portion of the immunoglobulin-expressing gene (i.e., rearranged transgene). Similarly, the artificial gene can encode an polypeptide that is identical or has substantial similarity to a polypeptide encoded by the sequenced portion of the rearranged transgene. The degeneracy of the genetic code allows the same polypeptide to be encoded by multiple nucleic acid sequences. It is sometimes desirable to change the nucleic acid sequence, for example to introduce restriction sites, change codon usage to reflect a particular expression system, or to remove a glycosylation site. In addition, changes in the hybridoma sequences may be introduced to change the characteristics (e.g., binding characteristics) of the immunoglobulin. For example, changes may be introduced, especially in the CDR regions of the heavy and light chain variable regions, to increase the affinity of the immunoglobulin for the predetermined antigen.

Methods for constructing an synthetic nucleic acids are well known. An entirely chemical synthesis is possible but in general, a mixed chemical-enzymatic synthesis is carried out in which chemically synthesized oligonucleotides are used in ligation reactions and/or in the polymerase chain reaction to create longer polynucleotides. In a most preferred embodiment, the polymerase chain reaction is carried out using overlapping primers chosen so that the result of the amplification is a DNA with the sequence desired for the artificial gene. The oligonucleotides of the present invention may be synthesized in solid phase or in solution. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of oligonucleotides by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers et al., U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage et al., *Tetrahedron Lett.*,

22:1859–1862; Matteucci et al., *J. Amer. Chem. Soc.*, 103:3185–3191 (1981); Caruthers et al., *Genetic Engineering*, 4:1–17 (1982); Jones, chapter 2, Atkinson et al., chapter 3, and Sproat et al., chapter 4, in Gait, ed. *Oligonucleotide Synthesis: A Practical Approach*, IRL Press, Washington, D.C. (1984); Froehler et al., *Tetrahedron Lett.*, 27:469–472 (1986); Froehler et al., *Nucleic Acids Res.*, 14:5399–5407 (1986); Sinha et al., *Tetrahedron Lett.*, 24:5843–5846 (1983); and Sinha et al., *Nucleic Acids Res.*, 12:4539–4557 (1984) which are incorporated herein by reference.

The artificial gene can introduced into a cell and expressed to produce an immunoglobulin polypeptide. The choice of cell type for expression will depend on many factors (e.g., the level of protein glycosylation desired), but cells capable of secreting human immunoglobulins will be preferred. Especially preferred cells include CHO cells and myeloma-derived cells such as the SP20 and NSO cell lines. Standard cell culture are well known and are also described in Newman, et al., *Biotechnology*, 10:1455–1460 (1992); Bebbington, et al., *Biotechnology*, 10:169–175 (1992); Cockett, et al., *Biotechnology*, 8:662–667 (1990); Carter, et al., *Biotechnology*, 10:163–167 (1992), each of which is incorporated herein by reference. Methods for introduction of nucleic acids, e.g., an artificial gene, are well known and include transfection (e.g., by electroporation or liposome-mediated) and transformation. Systems for expression of introduced genes are described generally in Sambrook et al., supra.

It is often desirable to express two immunoglobulin polypeptides (i.e., a heavy chain and a light chain) in the same cell so that an immunoglobulin (e.g., an IgG molecule) is produced in vivo. Accordingly it will sometimes be desirable to introduce two artificial genes (i.e., one encoding a heavy chain and one encoding a light chain) into a cell. (The two artificial genes can be introduced on a single vector). Alternatively, one artificial gene encoding one immunoglobulin polypeptide can be introduced into a cell that has been genetically engineered to express the other immunoglobulin polypeptide.

It will be apparent that as the cells into which the artificial gene is transfected propagate, the wholly synthetic nucleic acid portion of the artificial gene, will act as a template for replication and transcription. Nonetheless, the progeny genes will have originated from a synthetic nucleic acid (i.e., a polypeptide-encoding nucleic acid molecule that is synthesized in vitro by chemical or enzymatic methods that do not require a cell-derived template nucleic acid strand) and as used herein, are also considered artificial genes. Thus, the relationship of the synthetic portion of the artificial gene to the expressed transgene of the hybridoma is one in which there is an informational link (i.e., sequence information) but no direct physical link.

The invention also provides anti-CD4 monoclonal antibodies useful in therapeutic and diagnostic applications, especially the treatment of human disease. CD4 is a cell surface protein that is expressed primarily on thymocytes and T cells, and which is involved in T-cell function and MHC Class II recognition of antigen. Antibodies directed against CD4 act to reduce the activity of CD4 cells and thus reduce undesirable autoimmune reactions, inflammatory responses and rejection of transplanted organs.

Indeed, administration of anti-CD4 mABs has been shown to prevent (Wofsy, et al., *J. Exp. Med.*, 161:378–391 (1985)) or reverse (Wofsy, et al., *J. Immunol.*, 138:3247–3253 (1987), Waldor, et al., *Science*, 227:415–417 (1985)) autoimmune disease in animal models. Administration of murine or chimeric anti-CD4 mAbs to patients with rheumatoid arthritis has shown evidence of clinical benefit (Knox, et al., *Blood*, 77:20–30 (1991); Goldbery, et al., *J. Autoimmunity*, 4:617–630; Herzog, et al., *Lancet*, ii:1461–1462; Horneff, et al., *Arthritis Rheum.*, 34:129–140; Reiter, et al., *Arthritis Rheum.*, 34:525–536; Wending, et al., *J. Rheum.*, 18:325–327; Van der Lubbe, et al., *Arthritis Rheum.*, 38:1097–1106; Van der Lubbe, et al., *Arthritis Rheum.*, 36:1375–1379; Moreland, et al., *Arthritis Rheum.*, 36:307–318, and Choy, et al., *Arthritis and Rheumatism*, 39(1):52–56 (1996); all of which is incorporated herein by reference). In addition, as noted above, a chimeric anti-CD4 mAB has shown some clinical efficacy in patients with mycosis fungoides (Knox et al. (1991) Blood 77:20; which is incorporated herein by reference). Anti-CD4 antibodies are also discussed in Newman, et al., *Biotechnology*, 10:1455–1460 (1992), which is incorporated herein by reference.

EXPERIMENTAL EXAMPLES

Methods and Materials

Transgenic mice are derived according to Hogan, et al., "Manipulating the Mouse Embryo: A Laboratory Manual", Cold Spring Harbor Laboratory, which is incorporated herein by reference.

Embryonic stem cells are manipulated according to published procedures (Teratocarcinomas and embryonic stem cells: a practical approach, E. J. Robertson, ed., IRL Press, Washington, D.C., 1987; Zjilstra et al., *Nature* 342:435–438 (1989); and Schwartzberg et al., *Science* 246:799–803 (1989), each of which is incorporated herein by reference).

DNA cloning procedures are carried out according to J. Sambrook, et al. in Molecular Cloning: A Laboratory Manual, 2d ed., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference.

Oligonucleotides are synthesized on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer.

Hybridoma cells and antibodies are manipulated according to "Antibodies: A Laboratory Manual", Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), which is incorporated herein by reference.

Example 1

Genomic Heavy Chain Human Ig Transgene

This Example describes the cloning and microinjection of a human genomic heavy chain immunoglobulin transgene which is microinjected into a murine zygote.

Nuclei are isolated from fresh human placental tissue as described by Marzluff et al., "Transcription and Translation: A Practical Approach", B. D. Hammes and S. J. Higgins, eds., pp. 89–129, IRL Press, Oxford (1985)). The isolated nuclei (or PBS washed human spermatocytes) are embedded in a low melting point agarose matrix and lysed with EDTA and proteinase K to expose high molecular weight DNA, which is then digested in the agarose with the restriction enzyme NotI as described by M. Finney in Current Protocols in Molecular Biology (F. Ausubel, et al., eds. John Wiley & Sons, Supp. 4, 1988, Section 2.5.1).

The NotI digested DNA is then fractionated by pulsed field gel electrophoresis as described by Anand et al., *Nucl. Acids Res.* 17:3425–3433 (1989). Fractions enriched for the

NotI fragment are assayed by Southern hybridization to detect one or more of the sequences encoded by this fragment. Such sequences include the heavy chain D segments, J segments, p and γ1 constant regions together with representatives of all 6 V_H families (although this fragment is identified as 670 kb fragment from HeLa cells by Berman et al. (1988), supra., we have found it to be as 830 kb fragment from human placental an sperm DNA). Those fractions containing this NotI fragment (see FIG. **4**) are pooled and cloned into the NotI site of the vector pYACNN in Yeast cells. Plasmid pYACNN is prepared by digestion of pYAC-4 Neo (Cook et al., *Nucleic Acids Res.* 16: 11817 (1988)) with EcoRI and ligation in the presence of the oligonucleotide 5'-AAT TGC GGC CGC-3' (SEQ ID NO: 25).

YAC clones containing the heavy chain NotI fragment are isolated as described by Brownstein et al., *Science* 244:1348–1351 (1989), and Green et al., *Proc. Natl. Acad. Sci. USA* 87:1213–1217 (1990), which are incorporated herein by reference. The cloned NotI insert is isolated from high molecular weight yeast DNA by pulse field gel electrophoresis as described by M. Finney, op cit. The DNA is condensed by the addition of 1 mM spermine and microinjected directly into the nucleus of single cell embryos previously described.

Example 2

Genomic κ Light Chain Human Ig Transgene Formed by In Vivo Homologous Recombination

A map of the human κ light chain has been described in Lorenz et al., *Nucl. Acids Res.* 15:9667–9677 (1987), which is incorporated herein by reference.

A 450 kb XhoI to NotI fragment that includes all of C_κ, the 3' enhancer, all J segments, and at least five different V segments is isolated and microinjected into the nucleus of single cell embryos as described in Example 1.

Example 3

Genomic κ Light Chain Human Ig Transgene Formed by In Vivo Homologous Recombination

A 750 kb MluI to NotI fragment that includes all of the above plus at least 20 more V segments is isolated as described in Example 1 and digested with BssHII to produce a fragment of about 400 kb.

The 450 kb XhoI to NotI fragment plus the approximately 400 kb MluI to BssHII fragment have sequence overlap defined by the BssHII and XhoI restriction sites. Homologous recombination of these two fragments upon microinjection of a mouse zygote results in a transgene containing at least an additional 15–20 V segments over that found in the 450 kb XhoI/NotI fragment (Example 2).

Example 4

Construction of Heavy Chain Mini-locus

A. Construction of pGP1 and pGP2

pBR322 is digested with EcoRI and StyI and ligated with the following oligonucleotides to generate pGP1 which contains a 147 base pair insert containing the restriction sites shown in FIG. **8**. The general overlapping of these oligos is also shown in FIG. **9**.

The oligonucleotides are:

oligo-5'-CTT GAG CCC GCC TAA TGA GCG GGC TTT TTT TTG CAT ACT GCG GCC-3' (SEQ ID NO:26)

oligo-2 5'-GCA ATG GCC TGG ATC CAT GGC GCG CTA GCA TCG ATA TCT AGA GCT CGA GCA-3' (SEQ ID NO:27)

oligo-3 5'-TGC AGA TCT GAA TTC CCG GGT ACC AAG CTT ACG CGT ACT AGT GCG GCC GCT-3' (SEQ ID NO:28)

oligo-4 5'-AAT TAG CGG CCG CAC TAG TAC GCG TAA GCT TGG TAC CCG GGA ATT-3' (SEQ ID NO:29)

oligo-5 5'-CAG ATC TGC ATG CTC GAG CTC TAG ATA TCG ATG CTA GCG CGC CAT GGA TCC-3' (SEQ ID NO:30)

oligo-6 5'-AGG CCA TTG CGG CCG CAG TAT GCA AAA AAA AGC CCG CTC ATT AGG CGG GCT-3' (SEQ ID NO:31)

This plasmid contains a large polylinker flanked by rare cutting NotI sites for building large inserts that can be isolated from vector sequences for microinjection. The plasmid is based on pBR322 which is relatively low copy compared to the pUC based plasmids (pGP1 retains the pBR322 copy number control region near the origin of replication). Low copy number reduces the potential toxicity of insert sequences. In addition, pGP1 contains a strong transcription terminator sequence derived from trpA (Christie et al., *Proc. Natl. Acad. Sci. USA* 78:4180 (1981)) inserted between the ampicillin resistance gene and the polylinker. This further reduces the toxicity associated with certain inserts by preventing readthrough transcription coming from the ampicillin promoters.

Plasmid pGP2 is derived from pGP1 to introduce an additional restriction site (SfiI) in the polylinker. pGP1 is digested with MluI and SpeI to cut the recognition sequences in the polylinker portion of the plasmid.

The following adapter oligonucleotides are ligated to the thus digested pGP1 to form pGP2.

5' CGC GTG GCC GCA ATG GCC A 3' (SEQ ID NO:32)

5' CTA GTG GCC ATT GCG GCC A 3' (SEQ ID NO:33)

pGP2 is identical to pGP1 except that it contains an additional SfiI site located between the MluI and SpeI sites. This allows inserts to be completely excised with SfiI as well as with NotI.

B. Construction of pRE3 (Rat Enhancer 3')

An enhancer sequence located downstream of the rat constant region is included in the heavy chain constructs.

The heavy chain region 3' enhancer described by Petterson et al., *Nature* 344:165–168 (1990), which is incorporated herein by reference) is isolated and cloned. The rat IGH 3' enhancer sequence is PCR amplified by using the following oligonucleotides:

5' CAG GAT CCA GAT ATC AGT ACC TGA AAC AGG GCT TGC 3' (SEQ ID NO:34)

5' GAG CAT GCA CAG GAC CTG GAG CAC ACA CAG CCT TCC 3' (SEQ ID NO:35)

The thus formed double stranded DNA encoding the 3' enhancer is cut with BamHI and SphI and clone into BamHI/SphI cut pGP2 to yield pRE3 (rat enhancer 3').

C. Cloning of Human J-μ Region

A substantial portion of this region is cloned by combining two or more fragments isolated from phage lambda inserts. See FIG. **9**.

A 6.3 kb BamHI/HindIII fragment that includes all human J segments (Matsuda et al., *EMBO J.*, 7:1047–1051 (1988);

Ravetech et al. *Cell*, 27:583–591 (1981), which are incorporated herein by reference) is isolated from human genomic DNA library using the oligonucleotide GGA CTG TGT CCC TGT GTG ATG CTT TTG ATG TCT GGG GCC AAG (SEQ ID NO:36).

An adjacent 10 kb HindIII/BamH fragment that contains enhancer, switch and constant region coding exons (Yasui et al., *Eur. J. Immunol.* 19:1399–1403 (1989)) is similarly isolated using the oligonucleotide: CAC CAA GTT GAC CTG CCT GGT CAC AGA CCT GAC CAC CTA TGA (SEQ ID NO:37).

An adjacent 3' 1.5 kb BamHI fragment is similarly isolated using clone pMUM insert as probe (pMUM is 4 kb EcoRI/HindIII fragment isolated from human genomic DNA library with oligonucleotide:

CCT GTG GAC CAC CGC CTC CAC CTT CAT CGT CCT CTT CCT CCT (SEQ ID NO:38)

mu membrane exon 1) and cloned into pUC19.

pGP1 is digested with BamHI and BglII followed by treatment with calf intestinal alkaline phosphatase.

Fragments (a) and (b) from FIG. **9** are cloned in the digested pGP1. A clone is then isolated which is oriented such that 5' BamHI site is destroyed by BamHI/BglI fusion. It is identified as pMU (see FIG. **10**). pMU is digested with BamHI and fragment (c) from FIG. **9** is inserted. The orientation is checked with HindIII digest. The resultant plasmid pHIG1 (FIG. **10**) contains an 18 kb insert encoding J and Cμ segments.

D. Cloning of Cμ Region

pGP1 is digested with BamHI and HindIII is followed by treatment with calf intestinal alkaline phosphatase (FIG. **14**). The so treated fragment (b) of FIG. **14** and fragment (c) of FIG. **14** are cloned into the BamHI/HindIII cut pGP1. Proper orientation of fragment (c) is checked by HindIII digestion to form pCON1 containing a 12 kb insert encoding the Cμ region.

Whereas pHIG1 contains J segments, switch and μ sequences in its 18 kb insert with an SfiI 3' site and a SpeI 5' site in a polylinker flanked by NotI sites, will be used for rearranged VDJ segments. pCON1 is identical except that it lacks the J region and contains only a 12 kb insert. The use of pCON1 in the construction of fragment containing rearranged VDJ segments will be described hereinafter.

E. Cloning of γ-1 Constant Region (pREG2)

The cloning of the human γ-1 region is depicted in FIG. **16**.

Yamamura et al., *Proc. Natl. Acad. Sci. USA* 83:2152–2156 (1986) reported the expression of membrane bound human γ-1 from a transgene construct that had been partially deleted on integration. Their results indicate that the 3' BamHI site delineates a sequence that includes the transmembrane rearranged and switched copy of the gamma gene with a V-C intron of less than 5 kb. Therefore, in the unrearranged, unswitched gene, the entire switch region is included in a sequence beginning less than 5 kb from the 5' end of the first γ-1 constant exon. Therefore it is included in the 5' 5.3 kb HindIII fragment (Ellison et al., *Nucleic Acids Res.* 10:4071–4079 (1982), which is incorporated herein by reference). Takahashi et al., *Cell* 29: 671–679 (1982), which is incorporated herein by reference, also reports that this fragment contains the switch sequence, and this fragment together with the 7.7 kb HindIII to BamHI fragment must include all of the sequences we need for the transgene construct. An intronic sequence is a nucleotide sequence of at least 15 contiguous nucleotides that occurs in an intron of a specified gene.

Phage clones containing the γ-1 region are identified and isolated using the following oligonucleotide which is specific for the third exon of γ-1 (CH3).

5' TGA GCC ACG AAG AGC CTG AGG TCA AGT TCA ACT GGT ACG TGG 3' (SEQ ID NO:39)

A 7.7 kb HindIII to BglII fragment (fragment (a) in FIG. **11**) is cloned into HindIII/BglII cut pRE3 to form pREG1. The upstream 5.3 kb HindIII fragment (fragment (b) in FIG. **11**) is cloned into HindIII digested pREG1 to form pREG2. Correct orientation is confirmed by BamHI/SpeI digestion.

F. Combining Cγ and Cμ

The previously described plasmid pHIG1 contains human J segments and the Cμ constant region exons. To provide a transgene containing the Cμ constant region gene segments, pHIG1 was digested with SfiI (FIG. **10**). The plasmid pREG2 was also digested with SfiI to produce a 13.5 kb insert containing human Cγ exons and the rat 3' enhancer sequence. These sequences were combined to produce the plasmid pHIG3' (FIG. **12**) containing the human J segments, the human Cμ constant region, the human Cγ1 constant region and the rat 3' enhancer contained on a 31.5 kb insert.

A second plasmid encoding human Cμ and human Cγ1 without J segments is constructed by digesting pCON1 with SfiI and combining that with the SfiI fragment containing the human Cγ region and the rat 3' enhancer by digesting pREG2 with SfiI. The resultant plasmid, pCON (FIG. **12**) contains a 26 kb NotI/SpeI insert containing human Cμ, human γ1 and the rat 3' enhancer sequence.

G. Cloning of D Segment

The strategy for cloning the human D segments is depicted in FIG. **13**. Phage clones from the human genomic library containing D segments are identified and isolated using probes specific for diversity region sequences (Ichihara et al., *EMBO J.* 7:4141–4150 (1988)). The following oligonucleotides are used:

DXP1: 5'-TGG TAT TAC TAT GGT TCG GGG AGT TAT TAT AAC CAC AGT GTC-3' (SEQ ID NO:40)

DXP4: 5'-GCC TGA AAT GGA GCC TCA GGG CAC AGT GGG CAC GGA CAC TGT-3' (SEQ ID NO:41)

DN4: 5'-GCA GGG AGG ACA TGT TTA GGA TCT GAG GCC GCA CCT GAC ACC-3' (SEQ ID NO:42)

A 5.2 kb XhoI fragment (fragment (b) in FIG. **13**) containing DLR1, DXP1, DXP1, and DA1 is isolated from a phage clone identified with oligo DXP1.

A 3.2 kb XbaI fragment (fragment (c) in FIG. **13**) containing DXP4, DA4 and DK4 is isolated from a phage clone identified with oligo DXP4.

Fragments (b), (c) and (d) from FIG. **13** are combined and cloned into the XbaI/XhoI site of pGP1 to form pHIG2 which contains a 10.6 kb insert.

This cloning is performed sequentially. First, the 5.2 kb fragment (b) in FIG. **13** and the 2.2 kb fragment (d) of FIG. **13** are treated with calf intestinal alkaline phosphatase and cloned into pGP1 digested with XhoI and XbaI. The resultant clones are screened with the 5.2 and 2.2 kb insert. Half of those clones testing positive with the 5.2 and 2.2 kb inserts have the 5.2 kb insert in the proper orientation as determined by BamHI digestion. The 3.2 kb XbaI fragment from FIG. **13** is then cloned into this intermediate plasmid containing fragments (b) and (d) to form pHIG2. This

et al., *Biochemical Genetics*, 28:299–308 (1990)). This plasmid was used as the starting point for construction of the kappa targeting vector. The first step was to insert sequences homologous to the kappa locus 3' of the neo expression cassette.

Mouse kappa chain sequences (FIG. **20***a*) were isolated from a genomic phage library derived from liver DNA using oligonucleotide probes specific for the Cκ locus:

5' GGC TGA TGC TGC ACC AAC TGT ATC CAT CTT CCC ACC ATC CAG-3' (SEQ ID NO:58)

and for the Jκ5 gene segment:

5' CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA CGT AAG-3' (SEQ ID NO:59).

An 8 kb BglII/SacI fragment extending 3' of the mouse Cκ segment was isolated from a positive phage clone in two pieces, as a 1.2 kb BglII/SacI fragment and a 6.8 kb SacI fragment, and subcloned into BglII/SacI digested pGEM7 (KJ1) to generate the plasmid pNEO-K3' (FIG. **20***b*).

A 1.2 kb EcoRI/SphI fragment extending 5' of the J_κ region was also isolated from a positive phage clone. An SphI/XbaI/BglII/EcoRI adaptor was ligated to the SphI site of this fragment, and the resulting EcoRI fragment was ligated into EcoRI digested pNEO-K3', in the same 5' to 3' orientation as the neo gene and the downstream 3' kappa sequences, to generate pNEO-K5'3' FIG. **2***c*).

The Herpes Simplex Virus (HSV) thymidine kinase (TK) gene was then included in the construct in order to allow for enrichment of ES clones bearing homologous recombinants, as described by Mansour et al., *Nature* 336:348–352 (1988), which is incorporated herein by reference. The HSV TK cassette was obtained from the plasmid pGEM7 (TK), which contains the structural sequences for the HSV TK gene bracketed by the mouse pgk promoter and polyadenylation sequences as described above for pGEM7 (KJ1). The EcoRI site of pGEM7 (TK) was modified to a BamHI site and the TK cassette was then excised as a BamHI/HindIII fragment and subcloned into pGP1b to generate pGP1b-TK. This plasmid was linearized at the XhoI site and the XhoI fragment from pNEO-K3'3', containing the neo gene flanked by genomic sequences from 5' of Jκ and 3' of Cκ, was inserted into pGP1b-TK to generate the targeting vector J/C K1 (FIG. **20***d*). The putative structure of the genomic kappa locus following homologous recombination with J/C K1 is shown in FIG. **20***e*.

Generation and Analysis of ES Cells With Targeted Inactivation of a Kappa Allele

The ES cells used were the AB-1 line grown on mitotically inactive SNL76/7 cell feeder layers (McMahon and Bradley, *Cell* 62:1073–1085 (1990)) essentially as described (Robertson, E. J. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. E. J. Robertson, ed. (Oxford: IRL Press), p. 71–112). Other suitable ES lines include, but are not limited to, the E14 line (Hooper et al. (1987) *Nature* 326: 292–295), the D3 line (Doetschman et al. (1985) *J. Embryol. Exp. Morph.* 87: 27–45), and the CCE line (Robertson et al. (1986) *Nature* 323: 445–448). The success of generating a mouse line from ES cells bearing a specific targeted mutation depends on the pluripotence of the ES cells (i.e., their ability, once injected into a host blastocyst, to participate in embryogenesis and contribute to the germ cells of the resulting animal).

The pluripotence of any given ES cell line can vary with time in culture and the care with which it has been handled. The only definitive assay for pluripotence is to determine whether the specific population of ES cells to be used for targeting can give rise to chimeras capable of germline transmission of the ES genome. For this reason, prior to gene targeting, a portion of the parental population of AB-1 cells is injected into C57Bl/6J blastocysts to ascertain whether the cells are capable of generating chimeric mice with extensive ES cell contribution and whether the majority of these chimeras can transmit the ES genome to progeny.

The kappa chain inactivation vector J/C K1 was digested with NotI and electroporated into AB-1 cells by the methods described (Hasty et al., *Nature*, 350:243–246 (1991)). Electroporated cells were plated onto 100 mm dishes at a density of $1–2\times10^6$ cells/dish. After 24 hours, G418 (200μg/ml of active component) and FIAU (0.5 μM) were added to the medium, and drug-resistant clones were allowed to develop over 10–11 days. Clones were picked, trypsinized, divided into two portions, and further expanded. Half of the cells derived from each clone were then frozen and the other half analyzed for homologous recombination between vector and target sequences.

DNA analysis was carried out by Southern blot hybridization. DNA was isolated from the clones as described (Laird et al., *Nucl. Acids Res.* 19:4293 (1991)) digested with XbaI and probed with the 800 bp EcoRI/XbaI fragment indicated in FIG. **20***e* as probe A. This probe detects a 3.7 kb XbaI fragment in the wild type locus, and a diagnostic 1.8 kb band in a locus which has homologously recombined with the targeting vector (see FIGS. **20***a* and *e*). Of 901 G418 and FIAU resistant clones screened by Southern blot analysis, 7 displayed the 1.8 kb XbaI band indicative of a homologous recombination into one of the kappa genes. These 7 clones were further digested with the enzymes BglII, SacI, and PstI to verify that the vector integrated homologously into one of the kappa genes. When probed with the diagnostic 800 bp EcoRI/XbaI fragment (probe A), BglII, SacI, and PstI digests of wild type DNA produce fragments of 4.1, 5.4, and 7 kb, respectively, whereas the presence of a targeted kappa allele would be indicated by fragments of 2.4, 7.5, and 5.7 kb, respectively (see FIGS. **20***a* and *e*). All 7 positive clones detected by the XbaI digest showed the expected BglII, SacI, and PstI restriction fragments diagnostic of a homologous recombination at the kappa light chain. In addition, Southern blot analysis of an NsiI digest of the targeted clones using a neo specific probe (probe B, FIG. **20***e*) generated only the predicted fragment of 4.2 kb, demonstrating that the clones each contained only a single copy of the targeting vector.

Generation of Mice Bearing the Inactivated Kappa Chain

Five of the targeted ES clones described in the previous section were thawed and injected into C57Bl/6J blastocysts as described (Bradley, A. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. E. J. Robertson, ed. (Oxford: IRL Press), p. 113–151) and transferred into the uteri of pseudopregnant females to generate chimeric mice resulting from a mixture of cells derived from the input ES cells and the host blastocyst. The extent of ES cell contribution to the chimeras can be visually estimated by the amount of agouti coat coloration, derived from the ES cell line, on the black C57Bl/6J background. Approximately half of the offspring resulting from blastocyst injection of the targeted clones were chimeric (i.e., showed agouti as well as black pigmentation) and of these, the majority showed extensive (70 percent or greater) ES cell contribution to coat pigmentation. The AB1 ES cells are an XY cell line and a majority of these high percentage chimeras were male due to sex conversion of female embryos colonized by male ES

cells. Male chimeras derived from 4 of the 5 targeted clones were bred with C57BL/6J females and the offspring monitored for the presence of the dominant agouti coat color indicative of germline transmission of the ES genome. Chimeras from two of these clones consistently generated agouti offspring. Since only one copy of the kappa locus was targeted in the injected ES clones, each agouti pup had a 50 percent chance of inheriting the mutated locus. Screening for the targeted gene was carried out by Southern blot analysis of BglII-digested DNA from tail biopsies, using the probe utilized in identifying targeted ES clones (probe A, FIG. **20***e*). As expected, approximately 50 percent of the agouti offspring showed a hybridizing BglII band of 2.4 kb in addition to the wild-type band of 4.1 kb, demonstrating the germline transmission of the targeted kappa locus.

In order to generate mice homozygous for the mutation, heterozygotes were bred together and the kappa genotype of the offspring determined as described above. As expected, three genotypes were derived from the heterozygote matings: wild-type mice bearing two copies of a normal kappa locus, heterozygotes carrying one targeted copy of the kappa gene and one NT kappa gene, and mice homozygous for the kappa mutation. The deletion of kappa sequences from these latter mice was verified by hybridization of the Southern blots with a probe specific for J_κ (probe C, FIG. **20***a*). Whereas hybridization of the J_κ probe was observed to DNA samples from heterozygous and wild-type siblings, no hybridizing signal was present in the homozygotes, attesting to the generation of a novel mouse strain in which both copies of the kappa locus have been inactivated by deletion as a result of targeted mutation.

Example 10

Inactivation of the Mouse Heavy Chain Gene by Homologous Recombination

This example describes the inactivation of the endogenous murine immunoglobulin heavy chain locus by homologous recombination in embryonic stem (ES) cells. The strategy is to delete the endogenous heavy chain J segments by homologous recombination with a vector containing heavy chain sequences from which the J_H region has been deleted and replaced by the gene for the selectable marker neo.

Construction of a Heavy Chain Targeting Vector

Mouse heavy chain sequences containing the J_H region (FIG. **21***a*) were isolated from a genomic phage library derived from the D3 ES cell line (Gossler et al., *Proc. Natl. Acad. Sci. U.S.A.* 83:9065–9069 (1986)) using a J_H4 specific oligonucleotide probe:

5' ACT ATG CTA TGG ACT ACT GGG GTC AAG GAA CCT CAG TCA CCG-3' (SEQ ID NO:60)

A 3.5 kb genomic SacI/StuI fragment, spanning the J_H region, was isolated from a positive phage clone and subcloned into SacI/SmaI digested pUC18. The resulting plasmid was designated pUC18 J_H. The neomycin resistance gene (neo), used for drug selection of transfected ES cells, was derived from a repaired version of the plasmid pGEM7 (KJ1). A report in the literature (Yenofsky et al. (1990) *Proc. Natl. Acad. Sci. (U.S.A.)* 87: 3435–3439) documents a point mutation the neo coding sequences of several commonly used expression vectors, including the construct pMC1neo (Thomas and Cappechi (1987) *Cell* 51: 503–512) which served as the source of the neo gene used in pGEM7 (KJ1). This mutation reduces the activity of the neo gene product and was repaired by replacing a restriction fragment encompassing the mutation with the corresponding sequence from a wild-type neo clone. The HindIII site in the prepared pGEM7 (KJ1) was converted to a SalI site by addition of a synthetic adaptor, and the neo expression cassette excised by digestion with XbaI/SalI. The ends of the neo fragment were then blunted by treatment with the Klenow form of DNA poli, and the neo fragment was subcloned into the NaeI site of pUC18 J_H, generating the plasmid pUC18 J_H-neo (FIG. **21***b*).

Further construction of the targeting vector was carried out in a derivative of the plasmid pGP1b. pGP1b was digested with the restriction enzyme NotI and ligated with the following oligonucleotide as an adaptor:

5'-GGC CGC TCG ACG ATA GCC TCG AGG CTA TAA ATC TAG AAG AAT TCC AGC AAA GCT TTG GC-3' (SEQ ID NO:61)

The resulting plasmid, called pGMT, was used to build the mouse immunoglobulin heavy chain targeting construct.

The Herpes Simplex Virus (HSV) thymidine kinase (TK) gene was included in the construct in order to allow for enrichment of ES clones bearing homologous recombinants, as described by Mansour et al. (*Nature* 336, 348–352 (1988)). The HSV TK gene was obtained from the plasmid pGEM7 (TK) by digestion with EcoRI and HindIII. The TK DNA fragment was subcloned between the EcoRI and HindIII sites of pGMT, creating the plasmid pGMT-TK (FIG. **21***c*).

To provide an extensive region of homology to the target sequence, a 5.9 kb genomic XbaI/XhoI fragment, situated 5' of the J_H region, was derived from a positive genomic phage clone by limit digestion of the DNA with XhoI, and partial digestion with XbaI. As noted in FIG. **21***a*, this XbaI site is not present in genomic DNA, but is rather derived from phage sequences immediately flanking the cloned genomic heavy chain insert in the positive phage clone. The fragment was subcloned into XbaI/XhoI digested pGMT-TK, to generate the plasmid pGMT-TK-J_H5' (FIG. **21***d*).

The final step in the construction involved the excision from pUC18 J_H-neo of the 2.8 kb EcoRI fragment which contained the neo gene and flanking genomic sequences 3' of J_H. This fragment was blunted by Klenow polymerase and subcloned into the similarly blunted XhoI site of pGMT-TK-J_H5'. The resulting construct, J_HKO1 (FIG. **21***e*), contains 6.9 kb of genomic sequences flanking the J_H locus, with a 2.3 kb deletion spanning the J_H region into which has been inserted the neo gene. FIG. **21***f* shows the structure of an endogenous heavy chain gene after homologous recombination with the targeting construct.

Example 11

Generation and Analysis of Targeted ES Cells

AB-1 ES cells (McMahon and Bradley, *Cell* 62:1073–1085 (1990)) were grown on mitotically inactive SNL76/7 cell feeder layers essentially as described (Robertson, E. J. (1987) *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. E. J. Robertson, ed. (Oxford: IRL Press), pp. 71–112). As described in the previous example, prior to electroporation of ES cells with the targeting construct J_HKO1, the pluripotency of the ES cells was determined by generation of AB-1 derived chimeras which were shown capable of germline transmission of the ES genome.

The heavy chain inactivation vector J_HKO1 was digested with NotI and electroporated into AB-1 cells by the methods described (Hasty et al., *Nature* 350:243–246 (1991)). Electroporated cells were plated into 100 mm dishes at a density of $1–2\times10^6$ cells/dish. After 24 hours, G418 (200 mg/ml of active component) and FIAU (0.5 mM) were added to the medium, and drug-resistant clones were allowed to develop over 8–10 days. Clones were picked, trypsinized, divided into two portions, and further expanded. Half of the cells derived from each clone were then frozen and the other half analyzed for homologous recombination between vector and target sequences.

DNA analysis was carried out by Southern blot hybridization. DNA was isolated from the clones as described (Laird et al. (1991) *Nucleic Acids Res.* 19: 4293), digested with StuI and probed with the 500 bp EcoRI/StuI fragment designated as probe A in FIG. **21***f*. This probe detects a StuI fragment of 4.7 kb in the wild-type locus, whereas a 3 kb band is diagnostic of homologous recombination of endogenous sequences with the targeting vector (see FIGS. **21***a* and *f*). Of 525 G418 and FIAU doubly-resistant clones screened by Southern blot hybridization, 12 were found to contain the 3 kb fragment diagnostic of recombination with the targeting vector. That these clones represent the expected targeted events at the J_H locus (as shown in FIG. **21***f*) was confirmed by further digestion with HindIII, SpeI and HpaI. Hybridization of probe A (see FIG. **21***f*) to Southern blots of HindIII, SpeI, and HpaI digested DNA produces bands of 2.3 kb, >10 kb, and >10 kb, respectively, for the wild-type locus (see FIG. **21***a*), whereas bands of 5.3 kb, 3.8 kb, and 1.9 kb, respectively, are expected for the targeted heavy chain locus (see FIG. **21***f*). All 12 positive clones detected by the StuI digest showed the predicted HindIII, SpeI, and HpaI bands diagnostic of a targeted J_H gene. In addition, Southern blot analysis of a StuI digest of all 12 clones using a neo-specific probe (probe B, FIG. **21***f*) generated only the predicted fragment of 3 kb, demonstrating that the clones each contained only a single copy of the targeting vector.

Generation of Mice Carrying the J_H Deletion

Three of the targeted ES clones described in the previous section were thawed and injected into C57BL/6J blastocysts as described (Bradley, A. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (Oxford: IRL Press), p. 113–151) and transferred into the uteri of pseudopregnant females. The extent of ES cell contribution to the chimera was visually estimated from the amount of agouti coat coloration, derived from the ES cell line, on the black C57BL/6J background. Half of the offspring resulting from blastocyst injection of two of the targeted clones were chimeric (i.e., showed agouti as well as black pigmentation); the third targeted clone did not generate any chimeric animals. The majority of the chimeras showed significant (approximately 50 percent or greater) ES cell contribution to coat pigmentation. Since the AB-1 ES cells are an XY cell line, most of the chimeras were male, due to sex conversion of female embryos colonized by male ES cells. Males chimeras were bred with C57BL/6J females and the offspring monitored for the presence of the dominant agouti coat color indicative of germline transmission of the ES genome. Chimeras from both of the clones consistently generated agouti offspring. Since only one copy of the heavy chain locus was targeted in the injected ES clones, each agouti pup had a 50 percent chance of inheriting the mutated locus. Screening for the targeted gene was carried out by Southern blot analysis of StuI-digested DNA from tail biopsies, using the probe utilized in identifying targeted ES clones (probe A, FIG. **21***f*). As expected, approximately 50 percent of the agouti offspring showed a hybridizing StuI band of approximately 3 kb in addition to the wild-type band of 4.7 kb, demonstrating germline transmission of the targeted J_H gene segment.

In order to generate mice homozygous for the mutation, heterozygotes were bred together and the heavy chain genotype of the offspring determined as described above. As expected, three genotypes were derived from the heterozygote matings: wild-type mice bearing two copies of the normal J_H locus, heterozygotes carrying one targeted copy of the gene and one normal copy, and mice homozygous for the J_H mutation. The absence of J_H sequences from these latter mice was verified by hybridization of the Southern blots of StuI-digested DNA with a probe specific for J_H (probe C, FIG. **21***a*). Whereas hybridization of the J_H probe to a 4.7 kb fragment in DNA samples from heterozygous and wild-type siblings was observed, no signal was present in samples from the J_H-mutant homozygotes, attesting to the generation of a novel mouse strain in which both copies of the heavy chain gene have been mutated by deletion of the J_H sequences.

Example 12

Heavy Chain Minilocus Transgene

A. Construction of Plasmid Vectors for Cloning Large DNA Sequences

1. pGP1a

The plasmid pBR322 was digested with EcoRI and StyI and ligated with the following oligonucleotides:

oligo-42 5'-caa gag ccc gcc taa tga gcg ggc ttt ttt ttg cat act gcg gcc gct-3' (SEQ ID NO:62)

oligo-43 5'-aat tag cgg ccg cag tat gca aaa aaa agc ccg ctc att agg cgg gct-3' (SEQ ID NO:63)

The resulting plasmid, pGP1a, is designed for cloning very large DNA constructs that can be excised by the rare cutting restriction enzyme NotI. It contains a NotI restriction site downstream (relative to the ampicillin resistance gene, AmpR) of a strong transcription termination signal derived from the trpA gene (Christie et al., *Proc. Natl. Acad. Sci. USA* 78:4180 (1981)). This termination signal reduces the potential toxicity of coding sequences inserted into the NotI site by eliminating readthrough transcription from the AmpR gene. In addition, this plasmid is low copy relative to the pUC plasmids because it retains the pBR322 copy number control region. The low copy number further reduces the potential toxicity of insert sequences and reduces the selection against large inserts due to DNA replication. The vectors pGP1b, pGP1c, pGP1d, and pGP1f are derived from pGP1a and contain different polylinker cloning sites. The polylinker sequences are given below.

TABLE 1-continued

CCACCGCTGGTAGCGGTGGTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATC
CTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTAT
CAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAA
CTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACGTATCTCAGCGATCTGTCTATTTCGTTCATCCATAG
TTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATAC
CGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTG
GTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTA
ATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCA
GCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTC
CTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTA
CTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGC
GGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCA
TCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCA
CTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAA
ATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAA
GCATTTATCAGGGTTATTGTCTGATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTC
CGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATA
GGCGTATCACGAGGCCCTTTCGTCTTCAAG

Sequence of vector pGPe (SEQ ID NO:72)

B. Construction of IgM Expressing Minilocus Transgene, pIGM1

1. Isolation of J-μ Constant Region Clones and Construction of pJM1

A human placental genomic DNA library cloned into the phage vector λEMBL3/SP6/T7 (Clonctech Laboratories, Inc., Palo Alto, Calif.) was screened with the human heavy chain J region specific oligonucleotide:

oligo-1 5'-gga ctg tgt cec tgt gtg atg ctt ttg atg tct ggg gcc aag-3' (SEQ ID NO:73)

and the phage clone λ1.3 isolated. A 6 kb HindIII/KpnI fragment from this clone, containing all six J segments as well as D segment DHQ52 and the heavy chain J-μ intronic enhancer, was isolated. The same library was screened with the human μ specific oligonucleotide:

oligo-2 5'-cac caa gtt gac ctg cct ggt cac aga cct gac cac cta tga-3' (SEQ ID NO:74)

and the phage clone λ2.1 isolated. A 10.5 kb HindIII/XhoI fragment, containing the μ switch region and all of the μ constant region exons, was isolated from this clone. These two fragments were ligated together with KpnI/XhoI digested pNNO3 to obtain the plasmid pJM1.

2. pJM2

A 4 kb XhoI fragment was isolated from phage clone λ2.1 that contains sequences immediately downstream of the sequences in pJM1, including the so called Σμ element involved in δ-associated deletion of the μ in certain IgD expressing B-cells (Yasui et al., *Eur. J. Immunol.* 19:1399 (1989), which is incorporated herein by reference). This fragment was treated with the Klenow fragment of DNA polymerase I and ligated to XhoI cut, Klenow treated, pJM1. The resulting plasmid, pJM2 (FIG. **23**), had lost the internal XhoI site but retained the 3' XhoI site due to incomplete reaction by the Klenow enzyme. pJM2 contains the entire human J region, the heavy chain J-μ intronic enhancer, the μ switch region and all of the μ constant region exons, as well as the two 0.4 kb direct repeats, σμ and Σμ, involved in δ-associated deletion of the μ gene.

3. Isolation of D Region Clones and Construction of pDH1

The following human D region specific oligonucleotide:

oligo-4 5'-tgg tat tac tat ggt tcg ggg agt tat tat aac cac agt gtc-3' (SEQ ID NO:75)

was used to screen the human placenta genomic library for D region clones. Phage clones λ4.1 and λ4.3 were isolated. A 5.5 kb XhoI fragment, that includes the D elements D_{K1}, D_{N1}, and $D_{\Delta 2}$ (Ichihara et al., *EMBO J.* 7:4141 (1988)), was isolated from phage clone λ4.1. An adjacent upstream 5.2 kb XhoI fragment, that includes the D elements D_{XP1}, $D_{XP'1}$, and $D_{\Delta 1}$, was isolated from phage clone λ4.3. Each of these D region XhoI fragments were cloned into the SalI site of the plasmid vector pSP72 (Promega, Madison, Wis.) so as to destroy the XhoI site linking the two sequences. The upstream fragment was then excised with XhoI and SmaI, and the downstream fragment with EcoRV and XhoI. The resulting isolated fragments were ligated together with SalI digested pSP72 to give the plasmid pDH1. pDH1 contains a 10.6 kb insert that includes at least 7 D segments and can be excised with XhoI (5') and EcoRV (3').

4. pCOR1

The plasmid pJM2 was digested with Asp718 (an isoschizomer of KpnI) and the overhang filled in with the Klenow fragment of DNA polymerase I. The resulting DNA was then digested with ClaI and the insert isolated. This insert was ligated to the XhoI/EcoRV insert of pDH1 and XhoI/ClaI digested pGPe to generate pCOR1 (FIG. **24**).

5. pVH251

A 10.3 kb genomic HindIII fragment containing the two human heavy chain variable region segments V_H251 and V_H105 (Humphries et al., *Nature* 331:446 (1988), which is incorporated herein by reference) was subcloned into pSP72 to give the plasmid pVH251.

6. pIGM1

The plasmid pCOR1 was partially digested with XhoI and the isolated XhoI/SalI insert of pVH251 cloned into the upstream XhoI site to generate the plasmid pIGM1 (FIG. **25**). pIGM1 contains 2 functional human variable region segments, at least 8 human D segments all 6 human J_H segments, the human J-μ enhancer, the human σμ element, the human μ switch region, all of the human μ coding exons, and the human Σμ element, together with the rat heavy chain 3' enhancer, such that all of these sequence elements can be isolated on a single fragment, away from vector sequences, by digestion with NotI and microinjected into mouse embryo pronuclei to generate transgenic animals.

C. Construction of IgM and IgG Expressing Minilocus Transgene, pHC1

1. Isolation of γ Constant Region Clones

The following oligonucleotide, specific for human IgG constant region genes:

oligo-29 5'-cag cag gtg cac acc caa tgc cca tga gcc cag aca ctg gac-3' (SEQ ID NO:76)

was used to screen the human genomic library. Phage clones 129.4 and λ29.5 were isolated. A 4 kb HindIII fragment of

1296 carried the human IgM and IgG unrearranged transgene and was homozygous for mouse Ig heavy chain knockout. Mouse number 1299 carried the transgene on a non-knockout background, while mouse 1301 inherited neither of these sets of genes. Mouse 1297, another littermate, carried the human transgene and was hemizygous with respect to mouse heavy chain knockout. It was included as a non-immunized control.

The results demonstrate that both human IgG and IgM responses were developed to the hapten in the context of conjugation to protein. Human IgM also developed to the KLH molecule, but no significant levels of human IgG were present at this time point. In pre-immunization serum samples from the same mice, titers of human antibodies to the same target antigens were insignificant.

Example 20

This example demonstrates the successful immunization with a human antigen and immune response in a transgenic mouse of the present invention, and provides data demonstrating that nonrandom somatic mutation occurs in the variable region sequences of the human transgene.

Demonstration of Antibody Responses Comprising Human Immunoglobulin Heavy Chains Against a Human Glycoprotein Antigen

Transgenic mice used for the experiment were homozygous for functionally disrupted murine immunoglobulin heavy chain loci produced by introduction of a transgene at the joining (J) region (supra) resulting in the absence of functional endogenous (murine) heavy chain production. The transgenic mice also harbored at least one complete unrearranged human heavy chain mini-locus transgene, (HC1, supra), which included a single functional V_μ gene (V_μ251), human μ constant region gene, and human γ1 constant region gene. Transgenic mice shown to express human immunoglobulin transgene products (supra) were selected for immunization with a human antigen to demonstrate the capacity of the transgenic mice to make an immune response against a human antigen immunization. Three mice of the HC1-26 line and three mice of the HC1-57 line (supra) were injected with human antigen.

One hundred pg of purified human carcinoembryonic antigen (CEA) insolubilized on alum was injected in complete Freund's adjuvant on Day 0, followed by further weekly injections of alum-precipitated CEA in incomplete Freund's adjuvant on Days 7, 14, 21, and 28. Serum samples were collected by retro-orbital bleeding on each day prior to injection of CEA. Equal volumes of serum were pooled from each of the three mice in each group for analysis.

Titres of human μ chain-containing immunoglobulin and human γ chain-containing immunoglobulin which bound to human CEA immobilized on microtitre wells were determined by ELISA assay. Results of the ELISA assays for human μ chain-containing immunoglobulins and human γ chain-containing immunoglobulins are shown in FIGS. **38** and **39**, respectively. Significant human μ chain Ig titres were detected for both lines by Day 7 and were observed to rise until about Day 21. For human γ chain Ig, significant titres were delayed, being evident first for line HC1-57 at Day **14**, and later for line HC1-26 at Day 21. Titres for human γ chain Ig continued to show an increase over time during the course of the experiment. The observed human μ chain Ig response, followed by a plateau, combined with a later developing γ chain response which continues to rise is characteristic of the pattern seen with affinity maturation. Analysis of Day 21 samples showed lack of reactivity to an unrelated antigen, keyhole limpet hemocyanin (KLC), indicating that the antibody response was directed against CEA in a specific manner.

These data indicate that animals transgenic for human unrearranged immunoglobulin gene loci: (1) can respond to a human antigen (e.g., the human glycoprotein, CEA), (2) can undergo isotype switching ("class switching) as exemplified by the observed μ to γ class switch, and (3) exhibit characteristics of affinity maturation in their humoral immune responses. In general, these data indicate: (1) the human Ig transgenic mice have the ability to induce heterologous antibody production in response to a defined antigen, (2) the capacity of a single transgene heavy chain variable region to respond to a defined antigen, (3) response kinetics over a time period typical of primary and secondary response development, (4) class switching of a transgene-encoded humoral immune response from IgM to IgG, and (5) the capacity of transgenic animal to produce human-sequence antibodies against a human antigen.

Demonstration of Somatic Mutation in a Human Heavy Chain Transgene Minilocus

Line HC1-57 transgenic mice, containing multiple copies of the HC1 transgene, were bred with immunoglobulin heavy chain deletion mice to obtain mice that contain the HC1 transgene and contain disruptions at both alleles of the endogenous mouse heavy chain (supra). These mice express human mu and gamma1 heavy chains together with mouse kappa and lambda light chains (supra). One of these mice was hyperimmunized against human carcinoembryonic antigen by repeated intraperitoneal injections over the course of 1.5 months. This mouse was sacrificed and lymphoid cells isolated from the spleen, inguinal and mesenteric lymph nodes, and peyers patches. The cells were combined and total RNA isolated. First strand cDNA was synthesized from the RNA and used as a template for PCR amplification with the following 2 oligonucleotide primers:

149 5'-cta gct cga gtc cag gga gtc tgt gcc gag gtg cag ctg (g/a/t/c)-3' (SEQ ID NO:82)

151 5'-ggc gct cga gtt cca cga cac cgt cac cgg ttc-3' (SEQ ID NO:84)

These primers specifically amplify VH251/gamma1 cDNA sequences. The amplified sequences were digested with XhoI and cloned into the vector pNNO3. DNA sequence from the inserts of 23 random clones is shown in FIG. **40**; sequence variations from germline sequence are indicated, dots indicate sequence is identical to germline. Comparison of the cDNA sequences with the germline sequence of the VH251 transgene reveals that 3 of the clones are completely unmutated, while the other 20 clones contain somatic mutations. One of the 3 non-mutated sequences is derived from an out-of-frame VDJ joint. Observed somatic mutations at specific positions of occur at similar frequencies and in similar distribution patterns to those observed in human lymphocytes (Cai et al. (1992) *J. Exp. Med.* 176: 1073, incorporated herein by reference). The overall frequency of somatic mutations is approximately 1%; however, the frequency goes up to about 5% within CDR1, indicating selection for amino acid changes that affect antigen binding. This demonstrates antigen driven affinity maturation of the human heavy chain sequences.

Example 21

This example demonstrates the successful formation of a transgene by co-introduction of two separate polynucle-

human γ1 constant region genes. The transgenic line from which it originated was designated HC1-57 (supra).

One hundred μg of purified human carcinoembryonic antigen (CEA)(Cyrstal Chem, Chicago, Ill. or Scripps Labs, San Diego, Calif.) insolubilized on alum was injected in complete Freund's adjuvant on Day 0, followed by further weekly injections of alum-precipitated CEA in incomplete Freund's adjuvant on Days 7, 14, 21, and 28. An additional 20 μg of soluble CEA was administered intravenously on Day 83, followed by 50 μg alum-precipitated CEA in incomplete Freund's adjuvant on Day 92. Human heavy chain responses to CEA were confirmed in serum samples prior to fusion of spleen cells with myeloma cells. The animal was sacrificed on Day 95, the spleen removed and fused with P3X63-Ag8.653 mouse myeloma cells (ATCC CRL 1580, American Type Culture Collection, Rockville, Md.) using polyethylene glycol. Two weeks later, supernates from fusion wells were screened for the presence of antibodies specifically reactive with CEA, and which contained human heavy chain μ or γ constant region epitopes by ELISA. Briefly, purified human CEA was coated onto PVC microtitre plates at 2.5 μg/ml, and incubate with culture supernate diluted 1:4 or 1:5 in PBS, 0.5% Tween-20, 5% chicken serum. Plates were washed, followed by addition of horseradish peroxidase-conjugated goat antiserum specific for human IgG Fc or rabbit antiserum specific for human IgM Fc5Mu (Jackson ImmunoResearch, West Grove, Pa.). Presence of conjugate bound to captured antibody was determined, after further washing, by the addition of ABTS substrate. Two independent fusion wells were found to contain antibody with substantial binding to CEA. After cloning, both hybridomas were found to be positive for the presence of human μ chain and murine κ chain by ELISA. No mouse IgG or IgM were detected using similar assays.

Subcloning of the two independent parent hybridomas resulted in two clones, designated 92-09A-4f7-A5-2 and 92-09A-1D7-1-7-1. Both lines were deposited with the ATCC Patent Culture Depository under the Budapest Treaty and were assigned ATCC Designation HB 11307 and HB 11308, respectively. Culture supernatants from these cell lines were assessed for specificity by testing for reactivity to several purified target proteins using ELISA. As shown in FIG. 46, ELISA assays for determining the reactivity of the monoclonal antibodies to various antigens demonstrate that only CEA and the CEA-related antigen NCA-2 show significant reactivity, indicating the development of a restricted reactivity for the variable regions of the heterohybrid immunoglobulin molecules.

Example 23

This example demonstrates that a rearranged human VDJ gene encoded by a human Ig minilocus transgene may be transcribed as a transcript which includes an endogenous Ig constant region gene, for example by the mechanism of trans-switching, to encode a chimeric human/mouse Ig chain.

Identification of Trans-switch Transcripts Encoding Chimeric Human-mouse Heavy Chains

RNA was isolated from a hyperimmunized HC1 line 57 transgenic mouse homozygous for the endogenous heavy chain J segment deletion (supra). cDNA was synthesized according to Taylor et al. (1993) *Nucleic Acids Res.* 20: 6287, incorporated herein by reference, and amplified by PCR using the following two primers:

o-149 (human V_{HZ51})

5'-CTA GCT CGA GTC CAA GGA GTC TGT GCC GAG GTG CAG CTG (G,A,T,C)-3' (SEQ ID NO:82)

o-249 (mouse gamma):

5'-GGC GCT CGA GCT GGA CAG GG(A/C) TCC A(G/T)A GTT CCA-3' (SEQ ID NO:160)

Oligonucleotide o-149 is specific for the HC1-encoded variable gene segment V_{HZ51}, while o-249 hybridizes to both mouse and human gamma sequences with the following order of specificities:

mouse γ1=mouse γ2b=mouse γ3>mouse γ2a>>human γ1.

DNA sequences from 10 randomly chosen clones generated from the PCR products was determined and is shown in FIG. 47. Two clones comprised human VDJ and mouse γ1; four clones comprised human VDJ and mouse γ2b; and four clones comprised human VDJ and mouse γ3. These results indicate that in a fraction of the transgenic B cells, the transgene-encoded human VDJ recombined into the endogenous murine heavy chain locus by class switching or an analogous recombination.

Example 24

This example describes a method for screening a pool of hybridomas to discriminate clones which encode chimeric human/mouse Ig chains from clones which encode and express a human Ig chain. For example, in a pool of hybridoma clones made from a transgenic mouse comprising a human Ig heavy chain transgene and homozygous for a J region-disrupted endogenous heavy chain locus, hybridoma clones encoding trans-switched human VDJ-murine constant region heavy chains may be identified and separated from hybridoma clones expressing human VDJ-human constant region heavy chains.

Screening Hybridomas to Eliminate Chimeric Ig Chains

The screening process involves two stages, which may be conducted singly or optionally in combination: (1) a preliminary ELISA-based screen, and (2) a secondary molecular characterization of candidate hybridomas. Preferably, a preliminary ELISA-based screen is used for initial identification of candidate hybridomas which express a human VDJ region and a human constant region.

Hybridomas that show positive reactivity with the antigen (e.g., the immunogen used to elicit the antibody respouse in the transgenic mouse) are tested using a panel of monoclonal antibodies that specifically react with mouse μ, γ, κ, and λ, and human μ, γ, and κ. Only hybridomas that are positive for human heavy and light chains, as well as negative for mouse chains, are identified as candidate hybridomas that express human immunoglobulin chains. Thus, candidate hybridomas are shown to have reactivity with specific antigen and to possess epitopes characteristic of a human constant region.

RNA is isolated from candidate hybridomas and used to synthesize first strand cDNA. The first strand cDNA is then ligated to a unique single-stranded oligonucleotide of predetermined sequence (oligo-X) using RNA ligase (which ligates single-stranded DNA). The ligated cDNA is then amplified in two reactions by PCR using two sets of oligonucleotide primers. Set H (heavy chain) includes an oligo that specifically anneals to either human μ or human γ1 (depending on the results of the ELISA) and an oligo that anneals to the oligo-X sequence. This prevents bias against detection of particular V segments, including mouse V segments that may have trans-rearranged into the human minilocus. A second set of primers, Set L (light chain),

includes an oligo that specifically anneals to human κ and an oligo that anneals specifically to oligo-X. The PCR products are molecularly cloned and the DNA sequence of several are determined to ascertain whether the hybridoma is producing a unique human antibody on the basis of sequence comparison to human and murine Ig sequences.

Example 25

This example demonstrates production of a transgenic mouse harboring a human light chain (K) minilocus.

Human κ Minilocus Transgenic Mice

KC1

A 13 kb XhoI Jκ2-Kκ containing fragment from a phage clone (isolated from a human genomic DNA phage library by hybridization to a κ specific oligonucleotide, e.g., supra) was treated with Klenow enzyme and cloned into the Klenow treated HindIII site of pGP1d to produce pK-31. This destroyed the insert XhoI sites and positioned the unique polylinker derived XhoI site at the 5' end next to Jκ2. A unique polylinker derived ClaI site is located between this XhoI site and the inset sequences, while a unique polylinker derived SalI site is located at the 3' end of the insert. A 7.5 kb XhoI fragment, containing Jκ1 and upstream sequences, was also isolated from a human genomic DNA phage clone (isolated from a human genomic DNA phage library by hybridization to a κ specific oligonucleotide, e.g., supra). This 7.5 kb XhoI fragment was cloned into the SalI site of pSP72 (Promega, Madison, Wis.), thus destroying both XhoI sites and positioning a polylinker ClaI site 3' of Jκ1. Digestion of the resulting clone with ClaI released a 4.7 kb fragment containing Jk1 and 4.5 kb of upstream sequences. This 4.7 kb fragment was cloned into the ClaI site of pK-31 to create pKcor. The remaining unique 5' XhoI site is derived from polylinker sequences. A 6.5 kb XhoI/SalI DNA fragment containing the unrearranged human VκIII gene segment 65.8 (plasmid p65.8, EXAMPLE 21) was cloned into the XhoI site of pKcor to generate the plasmid pKC1. The NotI insert of pKC1 was microinjected into ½ day mouse embryos to generate transgenic mice. Two independent pKC1 derived transgenic lines were established and used to breed mice containing both heavy and light chain miniloci. These lines, KC1-673 and KC1-674, were estimated by Southern blot hybridization to contain integrations of approximately 1 and 10–20 copies of the transgenes respectively.

KC1e

The plasmid pMH81 (EXAMPLES 13 and 18) was digested with BamHI and HindIII to excise the 2.3 kb insert containing both the mouse and human heavy chain J-μ intronic enhancers. This fragment was Klenow treated, ligated to SalI linkers (New England Biolabs, Beverly, Mass.), and cloned into the unique 3' SalI site of pKC1 to generate the plasmid pKC1e. The NotI insert of pKC1e was microinjected into ½ day mouse embryos to generate transgenic mice. Four independent pKC1e derived transgenic lines were established and used to breed mice containing both heavy and light chain miniloci. These lines, KC1e-1399, KC1e-1403, KC1e-1527, and KC1e-1536, were estimated by Southern blot hybridization to contain integrations of approximately 20–50, 5–10, 1–5, and 3–5 copies of the transgene, respectively.

pKC2

A 6.8 kb XhoI/SalI DNA fragment containing the unrearranged human VκIII gene segment 65.5 (plasmid p65.5 g1, EXAMPLE 21) was cloned into the unique 5' XhoI site of pKC1 to generate the plasmid pKC2. This minilocus transgene contains two different functional VκIII gene segments. The NotI insert of pKC2 was microinjected into ½ day mouse embryos to generate transgenic mice. Five independent pKC2 derived transgenic lines were established and used to breed mice containing both heavy and light chain miniloci. These lines, KC2-1573, KC2-1579, KC2-1588, KC2-1608, and KC2-1610, were estimated by Southern blot hybridization to contain integrations of approximately 1–5, [1–50] 1–5, 50–100, and 5–20 copies of the transgene, respectively.

Example 26

This example shows that transgenic mice bearing the human κ transgene can make an antigen-induced antibody response forming antibodies comprising a functional human κ chain.

Antibody Responses Associated with Human Ig κ Light Chain

A transgenic mouse containing the HC1-57 human heavy chain and KC1e human κ transgenes was immunized with purified human soluble CD4 (a human glycoprotein antigen). Twenty μg of purified human CD4 (NEN Research products, Westwood, Mass.) insoluhilized by conjugation to polystyrene latex particles (Polysciences, Warrington, Pa.) was injected intraperitoneally in saline with dimethyldioctadecyl ammonium bromide (Calbiochem, San Diego, Calif.) on Day 0, followed by further injections on Day 20 and Day 34.

Retro-orbital bleeds were taken on Days 25 and 40, and screened for the presence of antibodies to CD4, containing human IgM or human IgG heavy chain by ELISA. Briefly, purified human CD4 was coated onto PVC microtitre plates at 2.5 μg/ml and incubated with culture supernate diluted 1:4/1:5 in PBS, 0.5% Tween-20, 5% chicken serum. Plates were washed, followed by addition of horseradish peroxidase-conjugated goat antiserum specific for human IgG Fc or rabbit antiserum specific for human IgM Fc5Mu (Jackson ImmunoResearch, West Grove, Pa.). Presence of conjugate bound to captured antibody was determined after further washing by addition of ABTS substrate. Human μ reactive with antigen was detected in both bleeds, while there was essentially undetectable γ reactivity. The Day 40 sample was also tested for antigen-reactive human κ chain using the same assay with goat anti-human κ peroxidase conjugate (Sigma, St. Louis, Mo.). CD4-binding κ reactivity was detected at this time point. The assay results are shown in FIG. 48.

Example 27

This example shows the successful generation of mice which are homozygous for functionally disrupted murine heavy and light chain loci (heavy chain and κ chain loci) and which concomitantly harbor a human heavy chain transgene and a human light chain transgene capable of productively rearranging to encode functional human heavy chains and functional human light chains. Such mice are termed "0011" mice, indicating by the two 0's in the first two digits that the mice lack functional heavy and light chain loci and indicating by the 1's in the second two digits that the mice are hemizygous for a human heavy chain transgene and a human light chain transgene. This example shows that such 0011 mice are capable of making a specific antibody response to a predetermined antigen, and that such an antibody response can involve isotype switching.

0011/0012 Mice: Endogenous Ig Knockout+Human Ig Transgenes

Mice which were homozygous for a functionally disrupted endogenous heavy chain locus lacking a functional J_H

region (designated JHD++ or JHΔ++) and also harboring the human HC1 transgene, such as the HC1-26 transgenic mouse line described supra, were interbred with mice homozygous for a functionally disrupted endogenous kappa chain locus lacking a functional J_{H} region (designated here as JKD++ or JKΔ++; see Example 9) to produce mice homozygous for functionally disrupted heavy chain and kappa chain loci (heavy chain/kappa chain knockouts), designated as JHD++/JKD++ and containing a HC1 transgene. Such mice were produced by interbreeding and selected on the basis of genotype as evaluated by Southern blot of genomic DNA. These mice, designated HC1-26+/JKD++/JHD++ mice, were interbred with mice harboring a human kappa chain transgene (lines KC2-1610, KC1e-1399, and KC1e-1527; see Example 25), and Southern blot analysis of genomic DNA was used to identify offspring mice homozygous for functionally disrupted heavy and light chain loci and also hemizygous for the HC1 transgene and the KC2 or KC1e transgene. Such mice are designated by numbers and were identified as to their genotype, with the following abbreviations: HC1-26+ indicates hemizygosity for the HC1-26 line human heavy chain minilocus transgene integration; JHD++ indicates homozygosity for J_{H} knockout; JKD++ indicates homozygosity for J_{κ} knockout; KC2-1610+ indicates hemizygosity for a KC2 human κ transgene integrated as in line KC2-1610; KC1e-1527+ indicates hemizygosity for a KC1e human κ transgene integrated as in line KC1e-1527; KC1e-1399+ indicates hemizygosity for a KC1e human κ transgene integrated as in line KC1e-1399.

The resultant individual offspring were each given a numerical designation (e.g., 6295, 6907, etc.) and each was evaluated for the presence of J_{H} knockout alleles, J_{κ} knockout alleles, HC1-26 transgene, and κ transgene (KC2 or KC1e) and determined to be either hemizygous (+) or homozygous (++) at each locus. Table 10 shows the number designation, sex, and genotypes of several of the offspring mice.

TABLE 10

ID No.	Sex	Ig Code	Genotype
6295	M	0011	HC1-26+;JHD++;JKD++;KC2-1610+
6907	M	0011	HC1-26+;JHD++;JKD++;KC1e-1527+
7086	F	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7088	F	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7397	F	0011	HC1-26+;JHD++;JKD++;KC1e-1527+
7494	F	0012	HC1-26+;JHD++;JKD++;KC2-1610++
7497	M	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7648	F	0011	HC1-26+;JHD++;JKD++;KC2-1610+
7649	F	0012	HC1-26+;JHD++;JKD++;KC2-1610++
7654	F	0011	HC1-26+;JHD++;JKD++;KC2-1610+
7655	F	0011	HC1-26+;JHD++;JKD++;KC2-1610+
7839	F	0011	HC1-26+;JHD++;JKD++;KC1e-1399+
7656	F	0001	HC1-26-;JHD++;JKD++;KC2-1610+
7777	F	1100	Col-2141-;JHD+;JKD+

We removed spleens from three 6 week old female mice. Mouse #7655 was determined by Southern blot hybridization to be hemizygous for the HC1 (line 26) and KC2 (line 1610) transgene integrations, and homozygous for the JH_ and Jκ_ targeted deletions of the mouse μ and κJ regions. Mouse #7656 was determincd by Southern blot hybridization to be hemizygous for the KC2 (line 1610) transgene integration and homozygous for the JH_ and Jk_ targeted deletions of the mouse μ κJ regions. Mouse #7777 was determined by Southern blot hybridization to be hemizygous for the JH_ and Jκ_ targeted deletions of the mouse μ and κJ regions. Because of the recessive nature of these deletions, this mouse should be phenotypically wild-type.

Expression of Endogenous Ig Chains in 0011 Mice

FACS analysis using a panel of antibodies reactive with either human μ, mouse μ, human κ, mouse κ, or mouse λ was used to sort lymphocytes explanted from (1) a wildtype mouse (7777), (2) a 0001 mouse homozygous for heavy chain and kappa knockout alleles and harboring a human light chain transgene (7656), and (3) a 0011 mouse homozygous for heavy chain and kappa knockout alleles and harboring a human light chain transgene and a human heavy chain transgene (7655).

We prepared single cell suspensions from spleen and lysed the red cells with NH_4Cl, as described by Mishell and Shiigi (Mishell, B. B. & Shiigi, S. M. (eds) *Selected Methods in Cellular Immunology*. W.H. Freeman & Co., New York, 1980). The lymphocytes are stained with the following reagents: propidium iodide (Molecular Probes, Eugene, Oreg.), FITC conjugated anti-human IgM (clone G20-127; Pharmingen, San Diego, Calif.), FITC conjugated anti-mouse IgM (clone R6-60.2; Pharmingen, San Diego, Calif.), phycoerythrin conjugated anti-human Igκ (clone HP6062; CalTag, South San Francisco, Calif.), FITC conjugated anti-mouse Igκ (clone R26-46; Pharmingen, San Diego, Calif.) FITC conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen, San Diego, Calif.), and Cy-Chrome conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen, San Diego, Calif.). We analyzed the stained cells using a FACScan flow cytometer and LYSIS II software (Becton Dickinson, San Jose, Calif.). Macrophages and residual red cells are excluded by gating on forward and side scatter. Dead cells are excluded by gating out propidium iodide positive cells. The flow cytometric data in FIGS. **49** and **50** confirms the Southern blot hybridization data and demonstrates that mouse #7655 expresses both human μ and human κ and relatively little if any mouse μ or mouse κ. Nevertheless a significant fraction of the B cells (about 70–80%) appear to express hybrid Ig receptors consisting of human heavy and mouse λ light chains.

FIG. **49** shows the relative distribution of B cells expressing human μ or mouse μ on the cell surface; 0011 mouse (7655) lymphocytes are positive for human μ but relatively lack mouse μ; 0001 mouse (7656) lymphocytes do not express much human μ or mouse μ; wildtype mouse (7777) lymphocytes express mouse μ but lack human μ.

FIG. **50** shows the relative distribution of B cells expressing human κ or mouse κ on the cell surface; 0011 mouse (7655) lymphocytes are positive for human κ but relatively lack mouse κ; 0001 mouse (7656) lymphocytes do not express much human κ or mouse κ; wildtype mouse (7777) lymphocytes express mouse κ but lack human κ.

FIG. **51** shows the relative distribution of B cells expressing mouse λ on the cell surface; 0011 mouse (7655) lymphocytes are positive for mouse λ; 0001 mouse (7656) lymphocytes do not express significant mouse λ; wildtype mouse (7777) lymphocytes express mouse λ but at a relatively lower level than the 0011 mouse (7655).

FIG. **52** shows the relative distribution of B cells positive for endogenous mouse λ as compared to human κ (transgene-encoded). The upper left panel shows the results of cells from a wildtype mouse possessing functional endogenous heavy and light chain alleles and lacking human transgene(s); the cells are positive for mouse lambda. The upper right panel shows cells from a mouse (#5822) having a κ knockout background (JKD++) and harboring the human κ transgene integration of the KC1e-1399 line; the cells are positive for human κ or mouse λ, in roughly proportional amounts. The lower left panel shows cells from a mouse

(#7132) having a κ knockout background (JKD++) and harboring the human κ transgene intergration of the KC2-1610 line; more cells are positive for mouse λ than for human κ, possibly indicating that the KC2-1610 transgene integration is less efficient than the KC1e-1399 transgene integration. The lower right panel shows cells from a mouse harboring a human κ minilocus transgene (KCo4) and lacking a functional endogenous murine κ allele. The data presented in FIG. **52** also demonstrates the variability of phenotypic expression between transgenes. Such variability indicates the desirability of selecting for individual transgenes and/or transgenic lines which express one or more desired phenotypic features resulting from the integrated transgene (e.g., isotype switching, high level expression, low murine Ig background). Generally, single or multiple transgene species (e.g., pKC1e, pKC2, KCo4) are employed separately to form multiple individual transgenic lines differing by: (1) transgene, (2) site(s) of transgene integration, and/or (3) genetic background. Individual transgenic lines are examined for desired parameters, such as: (1) capability to mount an immune response to a predetermined antigen, (2) frequency of isotypc switching within transgene-encoded constant regions and/or frequency of trans-switching to endogenous (e.g., murine) Ig constant region genes, (3) expression level of transgene-encoded immunoglobulin chains and antibodies, (4) expression level of endogenous (e.g., murine) immunoglobulin immunoglobulin sequences, and (5) frequency of productive VDJ and VJ rearrangement. Typically, the transgenic lines which produce the largest concentrations of transgene-encoded (e.g., human) immunoglobulin chains are selected; preferably, the selected lines produce about at least 40 μg/ml of transgenc-encoded heavy chain (e.g., human μ or human γ) in the serum of the transgenic animal and/or about at least 100 μg/ml of transgene-encoded light chain (e.g., human κ).

Mice were examined for their expression of human and murine immunoglobulin chains in their unimmunized serum and in their serum following immunization with a specific antigen, human CD4. FIG. **53** shows the relative expression of human μ, human γ, murine μ, murine γ, human κ, murine κ, and murine λ chains present in the serum of four separate unimmunized 0011 mice of various genotypes (nt=not tested); human κ predominates as the most abundant light chain, and human μ and murine γ (putatively a product of trans-switching) are the most abundant heavy chains, with variability between lines present, indicating the utility of a selection step to identify advantageous genotypic combinations that minimize expression of murine chains while allowing expression of human chains. Mice #6907 and 7088 show isotype switching (cis-switching within the transgene) from human μ to human γ.

FIG. **54** shows serum immunoglobulin chain levels for human μ (hμ), human γ (hγ), human κ (hκ), murine μ (mμ), murine γ (mγ), murine κ (mκ), and murine λ (mλ) in mice of the various 0011 genotypes.

Specific Antibody Response in 0011 Mice

An 0011 mouse (#6295) was immunized with an immunogenic dose of human CD4 according to the following immunization schedule: Day 0, intraperitoneal injection of 100 μl of CD4 mouse immune serum; Day 1, inject 20 μg of human CD4 (American Bio-Tech) on latex beads with DDA in 100 μl; Day 15 inject 20 μg of human CD4 (American Bio-Tech) on latex beads with DDA in 100 μl; Day 29 inject 20 μg of human CD4 (American Bio-Tech) on latex beads with DDA in 100 μl; Day 43 inject 20 μg of human CD4 (American Bio-Tech) on latex beads with DDA in 100 μl.

FIG. **55** shows the relative antibody response to CD4 immunization at 3 weeks and 7 weeks demonstrating the presence of human μ, human κ, and human γ chains in the anti-CD4 response. Human γ chains are present at significantly increased abundance in the 7 week serum, indicating that cis-switching within the heavy chain transgene (isotype switching) is occurring in a temporal relationship similar to that of isotype switching in a wildtype animal.

FIG. **56** shows a schematic compilation of various human heavy chain and light chain transgenes.

Example 28

This example provides for the targeted knockout of the murine λ light chain locus.

Targeted Inactivation of the Murine Lambda Light Chain Locus

Unlike the Ig heavy and kappa light chain loci, the murine VλJλ and Cλ gene segments are not grouped into 3 families arranged in a 5' to 3' array, but instead are interspersed. The most 5' portion consists of two V segments (Vλ2 and Vλ3) which are followed, proceeding in a 3' direction, by two constant region exons, each associated with its own J segment (Jλ2Cλ2 and the pseudogene Jλ4Cλ4). Next is the most extensively used V segment (Vλ1) which is followed by the second cluster of constant region exons (Jλ3Cλ3 and Jλ1Cλ1,). Overall the locus spans approximate 200 kb, with intervals of ~20–90 kb between the two clusters.

Expression of the lambda locus involves rearrangement of Vλ2 or Vλ3 predominantly to Jλ2 and only rarely further 3' to Jλ3 or Jλ1. Vλ1 can recombine with both Jλ3 and Jλ1. Thus the lambda locus can be mutated in order to fully eliminate recombination and expression of the locus.

The distance between the two lambda gene clusters makes it difficult to inactivate expression of the locus via the generation of a single compact targeted deletion, as was used in inactivating the murine Ig heavy and kappa light chain loci. Instead, a small single deletion which would eliminate expression lambda light chains spans approximately 120 kb, extending from Jλ2Cλ2 to Jλ1Cλ1 (FIG. **57**). This removes all of the lambda constant region exons as well as the Vλ1 gene segment, ensuring inactivation of the locus.

Replacement type targeting vectors (Thomas and Capecchi (1987) *op.cit*) are constructed in which the deleted 120 kb is replaced with the selectable marker genc, neo, in a PGK expression cassette. The marker is embedded within genomic lambda sequences flanking the deletion to provide homology to the lambda locus and can also contain the HSV-tk gene, at the end of onc of the regions of homology, to allow for enrichment for cells which have homologously integrated the vectors. Lambda locus genomic clone sequences are obtained by screening of a strain 129/Sv genomic phage library isogenic to the ES line being targeted, since the use of targeting vectors isogenic to the chromosomal DNA being targeted has been reported to enhance the efficiency of homologous recombination. Targeting vectors are constructed which differ in their lengths of homology to the lambda locus. The first vector (vector 1 in FIG. **58**) contains the marker gene flanked by total of approximately 8–12 kb of lambda locus sequences. For targeting events in which replacement vectors mediate addition or detection of a few kb of DNA this has been demonstrated to be a more than sufficient extent of homology (Hasty et al. (1991) *op.cit*; Thomas et al.(1992) *op.cit*.). Vectors with an additional approximately 40–60 kb of flanking lambda sequence are also constructed (vector 2 in FIG. **58**). Human Ig

miniloci of at least 80 kb are routinely cloned and propagated in the plasmid vector pGP1 (Taylor et al. (1993) *op.cit*).

An alternative approach for inactivation of the lambda locus employs two independent mutations, for example mutations of the two constant region clusters or of the two V region loci, in the same ES cell. Since both constant regions are each contained within ~6 kb of DNA, whereas one of the V loci spans ~19 kb, targeting vectors are constructed to independently delete the Jλ2Cλ2/Jλ4Cλ4 and the Jλ3Cλ3/Jλ1Cλ1 loci. As shown in FIG. **58**, each vector consists of a selectable marker (e.g., neo or pac) in a PGK expression cassette, surrounded by a total of ~8–12 kb of lambda locus genomic DNA blanking each deletion. The HSV-tk gene can be added to the targeting vectors to enrich for homologous recombination events by positive-negative selection. ES cells are targeted sequentially with the two vectors, such that clones are generated which carry a deletion of one of the constant region loci; these clones are then targeted sequentially with the two vectors, such that clones will be generated which carry a deletion of one of the constant region loci, and these clones are then targeted to generate a deletion of the remaining functional constant region cluster. Since both targeting events are thus being directed to the same cell, it is preferable to use a different selectable marker for the two targetings. In the schematic example shown in FIG. **58**, one of the vectors contains the neo gene and the other the pac (puromycin N-acetyl transferase) gene. A third potential dominant selectable marker is the hyg (hygromycin phosphotransferase) gene. Both the pac and hyg genes can be been inserted into the PGK expression construct successfully used for targeting the neo gene into the Ig heavy and kappa light chain loci. Since the two lambda constant region clusters are tightly linked, it is important that the two mutations reside on the same chromosome. There preferably is a 50% probability of mutating the same allele by two independent targeting events, and linkage of the mutations is established by their co-segregation during breeding of chimeras derived from the doubly targeted ES cells.

Example 29

This example provides for the targeted knockout of the murine heavy chain locus.

Targeted Inactivation of the Murine Heavy Chain Locus

A homologous recombination gene targeting transgene having the structure shown in FIG. **59** is used to delete at least one and preferably substantially all of the murine heavy chain locus constant region genes by gene targeting in ES cells. FIG. **59** shows a general schematic diagram of a targeting transgene. Segment (a) is a cloned genomic DNA sequence located upstream of the constant region gene(s) to be deleted [i.e.,] proximal to the J_H genes); segment (b) comprises a positive selection marker, such as pgk-neo; segment (c) is a cloned genomic DNA sequence located downstream of the constant region gene(s) to be deleted [i.e.,] distal to the constant region gene(s) and J_H genes); and segment (d), which is optional, comprises a negative selection marker gene (e.g., HSV-tk). FIG. **60** shows a map of the murine heavy chain locus as taken from *Immunoglobulin Genes*, Honjo, T, Alt, FW, and Rabbits TH (eds.) Academic Press, NY (1989) p. 129.

A targeting transgene having a structure according to FIG. **59**, wherein: (1) the (a) segment is the 11.5 kb insert of clone JH8.1 (Chen et al. (1993) *Int. Immunol.* 5: 647) or an equivalent portion comprising about at least 1–4 kb of sequence located upstream of the murine Cμ gene, (2) the (b) segment is pgk-neo as described supra, (3) the (c) segment comprises the 1674 bp sequence shown in FIG. **61** or a 4–6 kb insert isolated from a phage clone of the mouse Cα gene isolated by screening a mouse genomic clone library with the end-labeled oligonucleotide having the sequence:

5'-gtg ttg cgt gta tca gct gaa acc tgg aaa cag ggt gac cag-3'

and (4) the (d) segment comprises the HSV-tk expression cassette described supra.

Alternatively, a stepwise deletion of one or more heavy chain constant region genes is performed wherein a first targeting transgene comprises homology regions, i.e., segments (a) and (c), homologous to sequences flanking a constant region gene or genes, a first species of positive selection marker gene (pgk-neo), and an HSV-tk negative selection marker. Thus, the (a) segment can comprise a sequence of at least about 1–4 kb and homologous to a region located upstream of Cγ3 and the (c) segment can comprise a sequence of at least about 1–4 kb and homologous to a region located upstream of Cγ2a. This targeting transgene deletes the Cγ3, Cγ1, Cγ2b, and Cγ2a genes. This first targeting transgene is introduced into ES cells and correctly targeted recombinants are selected (e.g., with G418), producing a correctly targeted C region deletion. Negative selection for loss of the HSV-tk cassette is then performed (e.g., with ganciclovir or FIAU). The resultant correctly targeted first round C deletion recombinants have a heavy chain locus lacking the Cγ3, Cγ1, Cγ2b, and Cγ2a genes.

A second targeting transgene comprises homology regions, i.e., segments (a) and (c), homologous to sequences flanking a constant region gene or genes, a second species of positive selection marker gene different that the first species (e.g., gpt or pac), and an HSV-tk negative selection marker. Thus, the (a) segment can comprise a sequence of at least about 1–4 kb and homologous to a region located upstream of Cε and the (c) segment can comprise a sequence of at least about 1–4 kb and homologous to a region located upstream of Cα. This targeting transgene deletes the Cε and Cα genes.

This second targeting transgene is introduced into the correctly targeted C-region recombinant ES cells obtained from the first targeting event. Cells which are correctly targeted for the second knockout event (i.e., by homologous recombination with the second targeting transgene) are selected for with a selection drug that is specific for the second species of positive selection marker gene (e.g., mycophenolic acid to select for gpt; puromycin to select for pac). Negative selection for loss of the HSV-tk cassette is then performed (e.g., with ganciclovir or FIAU). These resultant correctly targeted second round C region recombinants have a heavy chain locus lacking the Cγ3, Cγ1, Cγ2b, Cγ2a, Cε, and Cα genes.

Correctly targeted first-round or second-round recombinant ES cells lacking one or more C region genes are used for blastocyst injections as described (supra) and chimeric mice are produced. Germline transmission of the targeted heavy chain alleles is established, and breeding of the resultant founder mice is performed to generate mice homozygous for C-region knockouts. Such C-region knockout mice have several advantages as compared to J_H knockout mice; for one example, C-region knockout mice have diminished ability (or completely lack the ability) to undergo trans-switching between a human heavy chain transgene and an endogenous heavy chain locus constant region, thus reducing the frequency of chimeric human/

mouse heavy chains in the transgenic mouse. Knockout of the murine gamma genes is preferred, although μ and delta are frequently also deleted by homologous targeting. C-region knockout can be done in conjunction with other targeted lesions in the endogenous murine heavy chain locus; a C-region deletion can be combined with a J_H knockout to preclude productive VDJ rearrangement of the murine heavy chain locus and to preclude or reduce trans-switching between a human heavy chain transgene and the murine heavy chain locus, among others. For some embodiments, it may be desirable to produce mice which specifically lack one or more C-region genes of the endogenous heavy chain locus, but which retain certain other C-region genes; for example, it may be preferable to retain the murine Cα gene to allow to production of chimeric human/mouse IgA by trans-switching, if such IgA confers an advantageous phenotype and does not substantially interfere with the desired utility of the mice.

Example 30

This example demonstrates ex vivo depletion of lymphocytes expressing an endogenous (murine) immunoglobulin from a lymphocyte sample obtained from a transgenic mouse harboring a human transgene. The lymphocytes expressing murine Ig are selectively depleted by specific binding to an anti-murine immunoglobulin antibody that lacks substantial binding to human immunoglobulins encoded by the transgene(s).

Ex Vivo Depletion of Murine Ig-Expressing B-cells

A mouse homozygous for a human heavy chain minilocus transgene (HC2) and a human light chain minilocus transgene (KCo4) is bred with a C57BL/6 (B6) inbred mouse to obtain 2211 mice (i.e., mice which: are homozygous for a functional endogenous murine heavy chain locus, are homozygous for a functional endogenous murine light chain locus, and which possess one copy of a human heavy chain transgene and one copy of a human light chain transgene). Such 2211 mice also express B6 major and minor histocompatibility antigens. These mice are primed with an immunogenic dose of an antigen, and after approximately one week spleen cells are isolated. B cells positive for murine Ig are removed by solid phase-coupled antibody-dependent cell separation according to standard methods (Wysocki et al. (1978) *Proc. Natl. Acad. Sci. (U.S.A.)* 75: 2844; MACS magnetic cell sorting, Miltenyi Biotec Inc., Sunnyvale, Calif.), followed by antibody-dependent complement-mediated cell lysis (*Selected Methods in Cellular Immunology*, Mishell B B and Shiigi S M (eds.), W.H. Freeman and Company, New York, 1980, pp. 211–212) to substantially remove residual cells positive for murine Ig. The remaining cells in the depleted sample (e.g., T cells, B cells positive for human Ig) are injected i.v., preferably together with additional anti-murine Ig antibody to deplete arising B cells, into a SCID/B6 or RAG/B6 mouse. The reconstituted mouse is then further immunized for the antigen to obtain antibody and affinity matured cells for producing hybridoma clones.

Example 31

Production of Fully Human Antibodies in Somatic Chimeras

A method is described for producing fully human antibodies in somatic chimeric mice. These mice are generated by introduction of embryonic stem (ES) cells, carrying human immunoglobulin (Ig) heavy and light chain transgenes and lacking functional murine Ig heavy and kappa light chain genes, into blastocysts from RAG-1 or RAG-2 deficient mice.

RAG-1 and RAG-2 deficient mice (Mombaerts et al. (1992) *Cell* 68: 869; Shinkai et al. (1992) *Cell* 68: 855) lack murine B and T cells due to an inability to initiate VDJ rearrangement and to assemble the gene segments encoding Igs and T cell receptors (TCR). This defect in B and T cell production can be complemented by injection of wild-type ES cells into blastocysts derived from RAG-2 deficient animals. The resulting chimeric mice produce mature B and T cells derived entirely from the injected ES cells (Chen et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 4528).

Genetic manipulation of the injected ES cells is used for introducing defined mutations and/or exogenous DNA constructs into all of the B and/or T cells of the chimeras. Chen et al. (1993), Proc. Natl. Acad. Sci. USA 90:4528–4532) generated ES cells carrying a homozygous inactivation of the Ig heavy chain locus, which, when injected into RAG blastocysts, produced chimeras which made T cells in the absence of B cells. Transfection of a rearranged murine heavy chain into the mutant ES cells results in the rescue of B cell development and the production of both B and T cells in the chimeras.

Chimeric mice which express fully human antibodies in the absence of murine Ig heavy chain or kappa light chain synthesis can be generated. Human Ig heavy and light chain constructs are introduced into ES cells homozygous for inactivation of both the murine Ig heavy and kappa light chain genes. The ES cells are then injected into blastocysts derived from RAG2 deficient mice. The resulting chimeras contain B cells derived exclusively from the injected ES cells which are incapable of expressing murine Ig heavy and kappa light chain genes but do express human Ig genes.

Generation of ES cells Homozygous for Inactivation of the Immunoglobulin Heavy and Kappa Light Chain Genes

Mice bearing inactivated Ig heavy and kappa light chain loci were generated by targeted deletion, in ES cells, of Ig J_H and J_K/C_K sequences, respectively according to known procedures (Chen et al. (1993) *EMBO J.* 12: 821; and Chen et al. (1993) *Int. Immunol. op.cit*). The two mutant strains of mice were bred together to generate a strain homozygous for inactivation of both Ig loci. This double mutant strain was used for derivation of ES cells. The protocol used was essentially that described by Robertson (1987, in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, p. 71–112, edited by E. J. Robertson, IRL Press). Briefly, blastocysts were generated by natural matings of homozygous double mutant mice. Pregnant females were ovariectomized on day 2.5 of gestation and the "delayed" blastocysts were flushed from the uterus on day 7 of gestation and cultured on feeder cells, to help maintain their undifferentiated state. Stem cells from the inner cell mass of the blastocysts, identifiable by their morphology, were picked, dissociated, and passaged on feeder cells. Cells with a normal karyotype were identified, and male cell lines will be tested for their ability to generate chimeras and contribute to the germ cells of the mouse. Male ES cells are preferable to female lines since a male chimera can produce significantly more offspring.

Introduction of Human Ig Genes Into Mouse Ig Heavy and Kappa Light Chain Deficient ES Cells

Human immunoglobulin heavy and light chain genes are introduced into the mutant ES cells as either minilocus

constructs, such as HC2 and KC-C04, or as YAC clones, such as J1.3P. Transfection of ES cells with human Ig DNAs is carried out by techniques such as electroporation or lipofection with a cationic lipid. In order to allow for selection of ES cells which have incorporated the human DNA, a selectable marker either is ligated to the constructs or is co-transfected with the constructs into ES cells. Since the mutant ES cells contain the neomycin phosphotransferse (neo) gene as a result of the gene targeting events which generated the Ig gene inactivations, different selectable markers, such as hygromycin phosphotransferase (hyg) or puromycin N-acetyl transferase (pac), are used to introduce the human Ig genes into the ES cells.

The human Ig heavy and light chain genes can be introduced simultaneously or sequentially, using different selectable markers, into the mutant ES cells. Following transfection, cells are selected with the appropriate selectable marker and drug-resistant colonies are expanded for freezing and for DNA analysis to verify and analyze the integration of the human gene sequences.

Generation of Chimeras

ES clones containing human Ig heavy and light chain genes are injected into RAG-2 blastocysts as described (Bradley, A. (1987), in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, p. 113–151, edited by E. J. Robertson, IRL Press) and transferred into the uteri of pseudopregnant females. Offspring are screened for the presence of human antibodies by ELISA assay of serum samples. Positive animals are used for immunization and the production of human monoclonal antibodies.

Example 32

This example describes the introduction, via homologous recombination in ES cells, of a targeted frameshift mutation into the murine heavy chain locus leading to a deletion of B cells which undergo switch recombination. The frameshifted mice are suitable hosts for harboring non-murine (e.g., human) transgenes encoding human sequence immunoglobulins.

The novel frameshifted mice can be used for expressing non-murine (e.g., human) sequence immunoglobulins encoded by heavy chain transgene(s) and/or light chain transgene(s), and for the isolation of hybridomas expressing class-switched, affinity matured, human sequence antibodies from introduced transgenes, among other uses. A frameshift is introduced into one of the four mouse JH gene segments and into the first exon of the mouse μ gene. The two introduced frameshift mutations compensate for each other thus allowing for the expression of fully functional murine μ heavy chain when a B cell uses the frameshifted JH for a functional VDJ joint. None of the other three JH segments can be used for functional VDJ joining because of the frameshift in μ, which is not compensated in the remaining JH genes. Alternatively, compensating frameshifts can be engineered into multiple murine JH genes.

A mouse homozygous for a compensated, frameshifted immunoglobulin heavy chain allele has an approximately physiological level of peripheral B cells, and an approximately physiological level of serum IgM comprising both murine and human μ. However, B cells recruited into germinal centers frequently undergo a class switch to a non-μ isotype. Such a class switch in B cells expressing the endogenous murine μ chain leads to the expression of a non-compensated frameshift mRNA, since the remaining non-μ C_H genes do not possess a compensating frameshift. The resulting B cells do not express a B cell receptor and are deleted. Hence, B cells expressing a murine heavy chain are deleted once they reach the stage of differentiation where isotype switching occurs. However, B cells expressing heavy chains encoded by a non-murine (e.g., human) transgene capable of isotype switching and which does not contain such isotype-restrictive frameshifts are capable of further development, including isotype switching and/or affinity maturation, and the like.

Therefore, the frameshifted mouse has an impaired secondary response with regard to murine heavy chain (μ) but a significant secondary response with regard to transgene-encoded heavy chains. If a heavy chain transgene that is capable of undergoing class switching is introduced into this mutant background, the non-IgM secondary response is dominated by transgene expressing B cells. It is thus possible to isolate affinity matured human sequence immunoglobulin expressing hybridomas from these frameshifted mice. Moreover, the frameshifted mice generally possess immunoprotective levels of murine IgM, which may be advantageous where the human heavy chain transgene can encode only a limited repertoire of variable regions.

For making hybridomas secreting human sequence monoclonal antibodies, transgenic mutant mice are immunized; their spleens fused with a myeloma cell line; and the resulting hybridomas screened for expression of the transgene encoded human non-μ isotype. Further, the frameshifted mouse may be advantageous over a JH deleted mouse because it will contain a functional μ switch sequence adjacent to a transcribed VDJ which serves as an active substrate for cis-switching (Gu et al. (1993) *Cell* 73: 1155); thus reducing the level of trans-switched B cells that express chimeric human/mouse antibodies.

Construction of Frameshift Vectors

Two separate frameshift vectors are built. One of the vectors is used to introduce 2 nucleotides at the 3' end of the mouse J4 gene segment, and one of the vectors is used to delete those same two nucleotides from the 5' end of exon 1 of the mouse μ gene.

1. JH Vector.

A 3.4 kb XhoI/EcoRI fragment covering the mouse heavy chain J region and the μ intronic enhancer is subcloned into a plasmid vector that contains a neomycin resistance gene as well as a herpes thymidine kinase gene under the control of a phosphoglycerate kinase promoter (tk/neo cassette; Hasty et al., (1991) *Nature* 350: 243). This clone is then used as a substrate for generating 2 different PCR fragments using the following oligonucleotide primers:

o-A1 5'-cca cac tct gca tgc tgc aga agc ttt tct gta-3' (SEQ ID NO:161)

o-A2 5'-ggt gac tga ggt acc ttg acc cca gta gtc cag-3' (SEQ ID NO:162)

o-A3 5'-ggt tac ctc agt cac cgt ctc ctc aga ggt aag aat ggc ctc-3' (SEQ ID NO:163)

o-A4 5'-agg ctc cac cag acc tct cta gac age aac tac-3' (SEQ ID NO:164)

Oligonucleotides o-A1 and o-A2 are used to amplify a 1.2 kb fragment which is digested with SphI and KpnI. Oligonucleotides o-A3 and o-A4 are used to amplify a 0.6 kb fragment which is digested with KpnI and XbaI. These two digested fragments are then cloned into SphI/XbaI digested plasmid A to produce plasmid B.

Plasmid B contains the 2 nucleotide insertion at the end of the J4 and, in addition, contains a new KpnI site upstream of the insertion. The KpnI site is used as a diagnostic marker for the insertion.

Additional flanking sequences may be cloned into the 5' XhoI site and the 3' EcoRI site of plasmid B to increase its homologous recombination efficiency. The resulting plasmid is then digested with SphI, or another restriction enzyme with a single site within the insert, and electroporated into embryonic stem cells which are then selected with G418 as described by Hasty et al. (1991) *op.cit*. Homologous recombinants are identified by Southern blot hybridization and then selected with FIAU as described by Hasty et al. to obtain deleted subclones which contain only the 2 base pair insertion and the new KpnI site in JH4. These are identified by Southern blot hybridization of KpnI digested DNA and confirmed by DNA sequence analysis of PCR amplified JH4 DNA.

The resulting mouse contains a JH4 segment that has been converted from the unmutated sequence:

. . . TGGGGTCAAGGAACCTCAGT-CACCGTCTCCTCAG_gtaagaatggcctctcc . . . TrpGlyGlnGlyThrSerValThrValSerSer (SEQ ID NOS:165 and 166, respectively)

to the mutant sequence:

. . . TGGGGTCAAGGTACCTCAGTCACCGTCTC-CTCAGAGgtaagaatggcctctcc . . . TrpGlyGlnGlyThrSerValThrValSerSerGlu (SEQ ID NOS:167 and 168, respectively)

μ Exon 1 Vector

Using similar in vitro mutagenesis methodology described above to engineer a two base pair insertion into the JH4 gene segment, PCR products and genomic subclones are assembled to create a vector containing a two base pair deletion at the 5' end of the first μ exon. In addition, to mark the mutation, a new XmnI site is also introduced downstream by changing an A to a G.

The sequence of the unmutated μ gene is:

. . . ctggtcctcagAGAGTCAGTCCTTCCCAAATGTCTT-CCCCCTCGTC . . . GluSerGlnSerPheProAsnValPheProLeuVal (SEQ ID NOS:169 and 170, respectively).

The sequence of the mutated μ gene is:

XmnI . . . ctggtcctcag_AGTCAGTCCTTCCC GAATGTCTTCCCCCTCGTC . . . SerGlnSerPheProAsnValPheProLeuVal (SEQ ID NOS:171 and 172, respectively).

The homologous recombination vector containing the mutant sequence is linearized and electroporated into an ES cell line containing the JH4 insertion. Homologous recombinants are identified from neomycin-resistant clones. Those homologous recombinants that contain the frameshift insertion on the same chromosome as the JH4 insertion are identified by Southern blot hybridization of KpnI/BamHI digested DNA. The JH4 insertion is associated with a new KpnI site that reduces the size of the J-μ intron containing KpnI/BamHI fragment from the wild type 11.3 kb to a mutant 9 kb. The resulting clones are then selected for deletion of the inserted tk/neo cassette using FIAU. Clones containing the mutant μ exon are identified by Southern blot hybridization of XmnI digested DNA. The mutation is confirmed by DNA sequence analysis of PCR amplified μ exon1 DNA.

Generation of Frameshifted Mice

The ES cell line containing both the two base pair insertion in JH4, and the two base pair deletion in μ exon 1, is then introduced into blastocyst stage embryos which are inserted into pseudopregnant females to generate chimeras. Chimeric animals are bred to obtain germline transmission, and the resulting animals are bred to homozygosity to obtain mutant animals homozygous for compensated frameshifted heavy chain loci and having impaired secondary humoral immune responses in B cells expressing murine heavy chains.

A human heavy chain transgene, such as for example pHC1 or pHC2 and the like, may be bred into the murine heavy chain frameshift background by crossbreeding mice harboring such a human transgene into mice having the frameshifted murine IgH locus. Via interbreeding and backcrossing, mice homozygous at the murine IgH locus for μ-compensated frameshifted murine IgH alleles (i.e., capable of compensated in-frame expression of only murine μ and not murine non-μ chains) and harboring at least one integrated copy of a functional human heavy chain transgene (e.g., pHC1 or pHC2) are produced. Such mice may optionally contain knockout of endogenous murine κ and/or λ loci as described supra, and may optionally comprise a human or other non-murine light chain transgene (e.g., pKC1e, pKC2, and the like).

Alternatively, the human transgene(s) (heavy and/or light) may comprise compensating frameshifts, so that the transgene J gene(s) contain a frameshift that is compensated by a frameshift in the transgene constant region gene(s). Transswitching to the endogenous constant region genes is uncompensated and produces a truncated or nonsense product; B cells expressing such uncompensated trans-switched immunoglobulins are selected against and depleted.

Example 33

Endogenous Heavy Chain Inactivation by D Region Ablation

This example describes a positive-negative selection homologous recombination vector for replacing the mouse germline immunoglobulin heavy chain D region with a nonfunctional rearranged VDJ segment. The resulting allele functions within a B cell as a normal non-productive allele, with the allele undergoing intra-allele heavy chain class switching, thereby reducing the level of trans-switching to an active transgene locus.

D Region Targeting Construct

An 8–15 kb DNA fragment located upstream of the murine D region is isolated and subcloned from a mouse strain 129 phage library using an oligonucleotide probe comprising approximately 50 consecutive nucleotides of the published sequence for the DFL16.1 segment listed in GenBank. DFL16.1 is the upstream D segment (i.e., proximal to the V region gene cluster and distal to the constant region gene cluster).

Similarly, a 9.5 kb BamHI fragment containing J_H3, JH4, $Eμ$, $Sμ$, and the first two coding exons of the μ constant region is isolated and subcloned from a mouse strain 129 genomic phage library.

A 5–10 kb rearranged VDJ is then isolated from a mouse hybridoma (any strain) and a synthetic linker containing a stop codon is inserted into the J segment. The stop linker within the J is preferable to an out-of-frame VDJ junction because of the possibility of V replacement rearrangements.

These three fragments are assembled together with a PGKneo positive selection cassette and a PGKHSVtk negative selection cassette to form a positive-negative selection vector for eliminating the mouse D region in 129-derived ES cells (e.g., AB1) by homologous recombination. The targeting vector is formed by ligating the 8–15 kb DNA fragment

specific antibodies we coated microtiter wells with human heavy chain isotype specific antibody (mouse MAb α human IgG1, clone HP6069, Calbiochem, La Jolla, Calif.; mouse MAb α human IgM, clone CH6, The Binding Site, Birmingham, UK) and developed with peroxidase conjugated antisera (horseradish peroxidase conjugated affinity purified fab fragment from polyclonal goat a human IgG(fc), cat #109-036-098; affinity purified horseradish peroxidase conjugated polyclonal rabbit α human IgM(fc), cat #309-035-095. Jackson Immuno Research, West Grove, Pa.). For detection of antigen-specific antibodies we coated microtiter wells with antigen and developed with peroxidase-conjugated human heavy chain isotype specific antisera. We detected bound peroxidase by incubation with hydrogen pcroxide and 2,2'-Azino-bis-(3-Ethylbenzthiazoline-6-Sulfonic Acid, Sigma Chem. Co., St. Louis, Mo.). The reaction product is measured by absorption at 415 nm, and corrected for absorption at 490 nm.

Flow Cytometry

We prepared single cell suspensions from spleen, bone marrow, and peritoneal cavity, and lysed red cells with NH_4Cl, as described by Mishell and Shiigi. The lymphocytes are stained with the following reagents: Phycoerythrin conjugated anti-mouse Igκ (clone X36; Becton Dickinson, San Jose, Calif.), FITC conjugated anti-mouse IgD (clone SBA 1, Southern Biotech, AL), FITC conjugated anti-mouse CD5 (clone 53-7.3; Becton Dickinson, San Jose, Calif.), FITC conjugated anti-mouse IgX (clone R26-46; Pharmingen, San Diego, Calif.), and Cy-Chrome conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen, San Diego, Calif.). We analyzed the stained cells using a FACScan flow cytometer and LYSIS II software (Becton Dickinson, San Jose, Calif.). Most macrophages, neutrophils, and residual red cells are excluded by gating on forward and side scatter.

Rescue of B Cell Compartment

In the peritoneal cavity of HC1 transgenic-JHD animals we find normal levels of $CD5^+$ B cells and approximately one-quarter the normal level of conventional $CD5^-$ B cells. The transgenic peritoneal $CD5^+$ B cells are similar to the so-called B-1 cells described in normal animals: they are larger than conventional B and T lymphocytes, they express lower levels of B220 than the conventional B cells found in the spleen, and they include a higher proportion of λ light chain expressing cells. Over 90% of the splenic B cells express κ, while up to 50% of the peritoneal B cells express λ. Thus, while the level of conventional B cells is uniformly reduced in all tissues, the level of B-1, which are reported to have a much greater capacity for self-renewal, appears to be normal in the HC1 transgenic-JHD animals.

Class Switching

In transgenic-JHD mice, repeated exposure to antigen results in the production of human γ1 antibodies as well as μ antibodies. We injected human CEA into transgenic-JHD mice at weekly intervals and monitored the serum levels of antigen-specific IgM and IgG1 over a period of four weeks (FIG. **63**). At one week there is a detectable IgM responsc but no IgG1 response. However, the IgG1 response is greater than the IgM response after two weeks, and it continues to increase while the IgM response remains relatively constant. This pattern—an initial IgM reaction followed by an IgG reaction—is typical of a secondary immune response; and it suggests that cis-acting sequences included in the transgene may be responding to cytokines that direct class switching.

We have considered three possible mechanisms for expression of non-μ isotypes, each of which have been discussed in the literature. These mechanisms are: alternative splicing, which does not involve deletion of the μ gene; "6-type" switching, which involved deletion of the μ gene via homologous recombination between flanking repeat sequences; and non-homologous recombination between switch regions. The results of our experiments, described below, are indicative of a switch region recombination model.

Two types of non-deletional alternative splicing mechanisms can be invoked to explain an isotype shift. First, it is possible that a single transcript covering both μ and γ1 is expressed from the transgenc; this transcript could be alternatively spliced in response to cytokines induced by exposure to antigen. Alternative, a cytokine induced sterile transcript initiating upstream of γ1 could be trans-spliced to the μ transcript. If either of these mechanisms were responsible for the expression of human γ1 sequences, then we would expect to be able to isolate hybridomas that express both μ and γ1. However, although we have screened several hundred hybridomas expressing either human μ or human γ1, we have not found any such double producer (μ^+, $\gamma 1^+$) hybridomas. This indicates that expression of γ1 is accompanied by deletion of the μ gene.

Deletion of the μ gene can be mediated by non-homologous recombination between the μ and γ1 switch regions, or by homologous recombination between the two flanking 400 bp direct repeats (Cμ and Σμ) that are included in the HC1 and HC2 transgenes. Deletional recombination between Cμ and Σμ has been reported to be responsible for the IgD^+, IgM^- phenotype of some human B cells. While the first mechanism, non-homologous switch recombination, should generate switch products of varying lengths, the second mechanism, Cμ/Σμ recombination, should always generate the same product. We performed a Southern blot analysis of genomic DNA isolated from three hybridomas (FIG. **64**A), one expressing μ and two expressing γ1. We find genomic rearrangements upstream of the transgene γ1 only in the two the γ1 switch regions (FIG. **64**B). Furthermore, neither of the observed structures is compatible with homologous recombination between Cμ and Σμ. Our results are therefore consistent with a model for γ1 isotype expression mediated by deletional non-homologous recombination between the transgene encoded μ and γ1 switch regions.

Trans-switching

In addition to human γ1, we find mouse γ in the serum of HC1 and HC2 transgenic-JHD mice. We have also obtained mouse γ expressing hybridomas from these animals. Because the non-transgenic homozygous JHD animals do not express detectable levels of mouse immunoglobulins, we attribute the expression of mouse γ in the HC1 and HC2 transgenic-JHD animals to the phenomenon of trans-switching. All of the transgenic hybridomas that we have analyzed express either mouse or human constant region sequences, but not both. It is therefore unlikely that a trans-splicing mechanism is involved. We used PCR amplification to isolate cDNA clones of trans-switch products, and determined the nucleotide sequence of 10 of the resulting clones (FIG. **65**). The 5' oligonucleotide in the PCR amplification is specific for the transgene encoded VH251, and the 3' oligonucleotide is specific for mouse γ1, γ2b, and γ3 sequences. We find examples of trans-switch products incorporating all three of these mouse constant regions.

Somatic Mutation

Approximately 1% of the nucleotides within the variable regions of the trans-switch products shown in FIG. **7** are not

germline encoded. This is presumably due to somatic mutation. Because the mutated sequence has been translocated to the endogenous locus, the cis-acting sequences directing these mutations could be located anywhere 3' of the mouse γ switch. However, as we discuss below, we also observe somatic mutation in VDJ segments that have not undergone such translocations; and this result indicates that sequences required by heavy chain somatic mutation are included in the transgene.

To determine if the HC1 and HC2 constructs include sufficient cis-acting sequences for somatic mutation to occur in the transgenic-JHD mice, we isolated and partially sequenced cDNA clones derived from two independent HC1 transgenic lines and one HC2 line. We find that some of the γ1 transcripts from transgenic-JHD mice contain V regions with extensive somatic mutations. The frequency of these mutated transcripts appears to increase with repeated immunizations. FIGS. 66A and 66B show two sets of cDNA sequences: one set is derived form an HC1 (line 26) transgenic-JHD mouse that we immunized with a single injection of antigen 5 days before we isolated RNA; the second set is derived from an HC1 (line 26) transgenic-JHD mouse that we hyperimmunized by injecting antigen on three different days beginning 5 months before we isolated RNA; the second set is derived from an HC1 (line 26) transgenic-JHD mouse that we hyperimmunized by injecting antigen on three different days beginning 5 months before we isolated RNA. Only 2 of the 13 V regions from the 5 day post-exposure mouse contain any non-germline encoded nucleotides. Each of these V's contains only a single nucleotide change, giving an overall somatic mutation frequency of less than 0.1% for this sample. In contrast, none of the 13 V sequences from the hyperimmunized animal are completely germline, and the overall somatic mutation frequency is 1.6%.

Comparison of μ and γ1 transcripts isolated from a single tissue sample shows that the frequency of somatic mutations is higher in transgene copies that have undergone a class switch. We isolated and partially sequenced 47 independent μ and γ1 cDNA clones from a hyperimmunized CH1 line 57 transgenic-JHD mouse (FIGS. 67A and 67B). Most of the μ cDNA clones are unmodified relative to the germline sequence, while over half of the γ1 clones contain multiple non-germline encoded nucleotides. The γ1 expressing cells are distinct from the μ expressing cells and, while the two processes are not necessarily linked, class switching and somatic mutation are taking place in the same subpopulation of B cells.

Although we do not find extensive somatic mutation of the VH251 gene in non-hyperimmunized CH1 transgenic mice, we have found considerable somatic mutation in VH56p1 and VH51p1 genes in a naive HC2 transgenic mouse. We isolated spleen and lymph node RNA from an unimmunized 9 week old female HC2 transgenic animal. We individually amplified γ1 transcripts that incorporate each of the four V regions in the HC2 transgene using V and γ1 specific primers. The relative yields of each of the specific PCR products were VH56p1>>VH51p1>VH4.21>VH251. Although this technique is not strictly quantitative, it may indicate a bias in V segment usage in the HC2 mouse. FIG. 68 shows 23 randomly picked γ1 cDNA sequences derived from PCR amplifications using an equimolar mix of all four V specific primers. Again we observe a bias toward VH56p1 (19/23 clones). In addition, the VH56p1 sequences show considerable somatic mutation, with an overall frequency of 2.1% within the V gene segment. Inspection of the CDR3 sequences reveals that although 17 of the 19 individual VH56p1 clones are unique, they are derived from only 7 different VDJ recombination events. It thus appears that the VH56p1 expressing B cells are selected, perhaps by an endogenous pathogen or self antigen, in the naive animal. It may be relevant that this same gene is over-represented in the human fetal repertoire.

Summary

Upstream cis-acting sequences define the functionality of the individual switch regions, and are necessary for class switching. Our observation—that class switching within the HC1 transgene is largely confined to cells involved in secondary response, and does not occur randomly across the entire B cell population—suggests that the minimal sequences contained with the transgene are sufficient. Because the γ sequences included in this construct begin only 116 nucleotides upstream of the start site of the γ1 sterile transcript, the switch regulatory region is compact.

Our results demonstrate that these important cis-acting regulatory elements are either closely linked to individual γ genes, or associated with the 3' heavy chain enhancer included in the HC1 and HC2 transgenes. Because the HC1 and HC2 inserts undergo transgene-autonomous class switching—which can serve as a marker for sequences that are likely to have been somatically mutated—we were able to easily find hypermutated transcripts that did not originate from translocations to the endogenous locus. We found somatically mutated γ transcripts in three independent transgenic lines (two HC1 lines and one HC2 line). It is therefore unlikely that sequences flanking the integration sites of the transgene affect this process; instead, the transgene sequences are sufficient to direct somatic mutation.

Example 36

This example describes the generation of hybridomas from mice homozygous for an inactivated endogenous immunoglobulin locus and containing transgene sequences encoding a human sequence heavy chain and human sequence light chain. The hybridomas described secrete monoclonal antibodies comprising a human sequence heavy chain and a human sequence light chain and bind to a predetermined antigen expressed on T lymphocytes. The example also demonstrates the capacity of the mice to make a human sequence antibody in response to a human-derived immunogen, human CD4, and the suitability of such mice as a source for making hybridomas secreting human sequence monoclonal antibodies reactive with human antigens.

A. Generation of Human Ig Monoclonal Antibodies Derived From HC1 Transgenic Mice Immunized With a Human CD4 Antigen

A transgenic mouse homozygous for a functionally disrupted J_H locus and harboring a transgene capable of rearranging to encode a human sequence heavy chain and a transgene capable of rearranging to encode a human sequence light chain was immunized. The genotype of the mouse was HC1-26*KC1e-1536*$J_HD^{-/-}J_L D^{-}$, indicating homozygosity for murine heavy chain inactivation and the presence of germline copies of the HC1 human sequence heavy chain transgene and the KC1e human sequence light chain transgene.

The mouse was immunized with a variant of the EL4 cell line (ATCC) expressing a mouse-human hybrid CD4 molecule encoded by a stably transfected polynucleotide. The expressed CD4 molecule comprises a substantially human-like CD4 sequence. Approximately 5×10^6 cells in 100 μl of

PBS accompanied by 100 μl of Complete Freund's Adjuvant (CFA) were introduced into the mouse via intraperitoneal injection on Day 0. The inoculation was repeated on Days 7, 14, 21, 28, 60, and 77, with test bleeds on Days 18, 35, and 67. The spleen was removed on Day 81 and approximately 7.2×10^7 spleen cells were fused to approximately 1.2×10^7 fusion partner cells (P3x63Ag8.653 cell line; ATCC) by standard methods (PEG fusion) and cultured in RPMI 1640 15% FCS, 4 mM glutamine, 1 mM sodium pyruvate plus HAT and PSN medium. Multiple fusions were performed.

Hybridomas were grown up and supernatants were tested with ELISA for binding to a commercial source of purified recombinant soluble human sequence CD4 expressed in CHO cells (American Bio-Technologies, Inc. (ABT), Cambridge, Mass.) and/or CD4 obtained from NEN-DuPont. The ABT sample contained a purified 55 kD human CD4 molecule comprised the V_1 through V_3 domains of human CD4. The recombinant human sequence CD4 (produced in CHO-K1 cells) was adsorbed to the assay plate and used to capture antibody from hybridoma supernatants, the captured antibodies were then evaluated for binding to a panel of antibodies which bind either human μ, human κ, human γ, murine μ, or murine κ.

One hybridoma was subcloned from its culture plate well, designated 1F2. The 1F2 antibody bound to the ABT CD4 preparation, was positive for human μ and human κ, and was negative for human γ, mouse γ, and mouse κ.

B. Generation of Human Ig Monclonal Antibodies Derived From HC2 Transgenic Micc Immunized With Human CD4 and Human IgE

The heavy chain transgene, HC2, is shown in FIG. **56** and has been described supra (see, Example 34).

The human light chain transgene, KCo4, depicted in FIG. **56** is generated by the cointegration of two individually cloned DNA fragments at a single site in the mouse genome. The fragments comprise 4 functional Vκ segments, 5J segments, the Cκ exon, and both the intronic and down-stream enhancer elements (see Example 21) (Meyer and Neuberger (1989), *EMBO J.* 8:1959–1964; Judde and Max (1992), *Mol. Cell Biol.* 12:5206–5216). Because the two fragments share a common 3 kb sequence (see FIG. **56**), they can potentially integrate into genomic DNA as a contiguous 43 kb transgene, following homologous recombination between the overlapping sequences. It has been demonstrated that such recombination events frequently occur upon microinjection of overlapping DNA fragments (Piepcr et al. (1992), *Nucleic Acids Res.* 20:1259–1264). Co-injected DNA's also tend to co-integrate in the zygote, and the sequences contained within the individually cloned fragments would subsequently be jointed by DNA rearrangement during B cell development. Table 11 shows that transgene inserts from at least 2 of the transgenic lines are functional. Examples of VJ junctions incorporating each of the 4 transgene encoded V segments, and each of the 5J segments, are represented in this set of 36 clones.

TABLE 11

line	$V_κ5.5$	$V_κ8.8$	$V_κ45.15$	$V_κ5.3$	$J_κ1$	$J_κ2$	$J_κ3$	$J_κ4$	$J_κ5$
#4436	0	11	4	3	14	1	0	2	1
#4437	1	3	7	7	5	2	1	7	3

Human light chain V and J segment usage in KCo4 transgenic mice. The table shows the number of PCR clones, amplified from cDNA derived from two transgenic lines, which contain the indicated human kappa sequences. cDNA was synthesized using spleen RNA isolated from individual KC04 transgenic mice (mouse #8490, 3 mo., male, KC04 line 4437; mouse #8867, 2.5 mo., female, KC04 line 4436). The cDNA was amplified by PCR using a Cκ specific oligonucleotide, 5' TAG AAG GAA TTC AGC AGG CAC ACA ACA GAG GCA GTT CCA 31 (SEQ ID NO:173), and a 1:3 mixture of the following 2 Vκ specific oligonucleotides: 5' AGC TTC TCG AGC TCC TGC TGC TCT GTT TCC CAG GTG CC 31 (SEQ ID NO:174) and 5' CAG CTT CTC GAG CTC CTG CTA CTC TGG CTC (C,A)CA GAT ACC 31 (SEQ ID NO:175). The PCR product was digested with XhoI and EcoRI, and cloned into a plasmid vector. Partial nucleotide sequences were determined by the dideoxy chain termination method for 18 randomly picked clones from each animal. The sequences of each clone were compared to the germline sequence of the unrearranged transgene.

Twenty-three light chain minilocus positive and 18 heavy chain positive mice developed from the injected embryos. These mice, and their progeny, were bred with mice containing targeted mutations in the endogenous mouse heavy (strain JHD) and κ light chain loci (strain JCKD) to obtain mice containing human heavy and κ light chain in the absence of functional mouse heavy and κ light chain loci. In these mice, the only mouse light chain contribution, if any, is from the mouse λ locus.

Table 12 shows that somatic mutation occurs in the variable regions of the transgene-encoded human heavy chain transcripts of the transgenic mice. Twenty-three cDNA clones from a HC2 transgenic mouse were partially sequenced to determine the frequency of non-germline encoded nucleotides within the variable region. The data include only the sequence of V segment codons 17–94 from each clone, and does not include N regions. RNA was isolated from the spleen and lymph node of mouse 5250 (HC2 line 2550 henizygous, JHD homozygous). Single-stranded cDNA was synthesized and γ transcripts amplified by PCR as described [references]. The amplified cDNA was cloned into plasmid vectors, and 23 randomly picked clones were partially sequenced by the dideoxy chain-termination method. The frequency of PCR-introduced nucleotide changes is estimated from constant region sequence as <0.2%.

TABLE 12

The Variable Regions of Human γ Transcripts in HC2 Transgenic Mice Contain Non-Germline-Encoded Nucleotides

VH Segment	Number of clones	Number of non-germline encoded nucleotides	Frequency of non-germline-encoded nucleotides (%)
VH251	0		—
VH56P1	10	100	2.1
VH51P1	1	5	2.0
VH4.21	3	0	0.0

Flow Cytometry

We analyzed the stained cells using a FACScan flow cytometer and LYSIS II software (Becton Dickinson, San Jose, Calif.). Spleen cells were stained with the following reagents: propidium iodide (Molecular Probes, Eugene, Oreg.), phycoerythrin conjugated α-human Igκ (clone HP6062; Caltag, S. San Francisco, Calif.), phycocrythrin conjugated α-mouse Igκ (clone λ36; Becton Dickinson, San Jose, Calif.), FITC conjugated α-mouse Igλ (clone R26-46;

Pharmingen, San Diego, Calif.), FITC conjugated α-mouse IgD (clone R6-60.2; Pharmingen, San Diego, Calif.), FITC conjugated α-human IgM (clone G20-127; Pharmingen, San Diego, Calif.), and Cy-Chrome conjugated α-mouse B220 (clone RA3-6B2; Pharmingen, San Diego, Calif.).

Expression of Human Ig Transgenes

FIG. **69** shows a flow cytometric analysis of spleen cells from KCo4 and HC2 mice that are homozygous for both the JHD and JCKD mutations. The human sequence HC2 transgene rescued B cell development in the JHD mutant background, restoring the relative number of B220$^+$ cells in the spleen to approximately half that of a wild type animal. These B cells expressed cell surface immunoglobulin receptors that used transgene encoded heavy chain. The human KCo4 transgene was also functional, and competed successfully with the intact endogenous λ light chain locus. Nearly 95% of the splenic B cells in JHD/JCKD homozygous mutant mice that contain both heavy and light chain human transgenes (double transgenic) expressed completely human cell surface IgMK.

Serum Ig levels were determined by ELISA done as follows: human u: microtiter wells coated with mouse Mab a human IgM (clone CH6, The Binding Site, Birmingham, UK) and developed with peroxidase conjugated rabbit a human IgM(fc) (cat #309-035-095, Jackson Immuno Research, West Grove, Pa.). Human γ: microtiter wells coated with mouse MAb α human IgG1 (clone HP6069, Calbiochem, La Jolla, Calif.) and developed with peroxidase conjugated goat α human IgG(fc) (cat #109-036-098, Jackson Immuno Research, West Grove, Pa.). Human κ: microtiter wells coated with mouse Mab α human Igκ (cat #0173, AMAC, Inc. Igκ (cat #A7164, Sigma Chem. Co., St. Louis, Mo.). Mouse γ: microtiter wells coated with goat a mouse IgG (cat #115-006-071, Jackson Immuno Research, West Grove, Pa.). Mouse λ: microtiter wells coated with rat MAb α mouse Igλ (cat #02171D, Pharmingen, San Diego, Calif.) and developed with peroxidase conjugated rabbit a mouse IgM(fc) (cat #309-035-095, Jackson Immuno Research, West Grove, Pa.). Bound peroxidase is detected by incubation with hydrogen peroxide and 2,2'-Azino-bis-(3-Ethylbenzthiazoline-6-Sulfonic Acid, Sigma Chem. Co., St. Louis, Mo.). The reaction product is measured by absorption at 415 nm.

The double transgenic mice also express fully human antibodies in the serum. FIG. **70** shows measured serum levels of immunoglobulin proteins for 18 individual double transgenic mice, homozygous for endogenous heavy and kappa light chain inactivations, derived from several different transgenic founder animals. We found detectable levels of human μ, γ1, and κ. We have shown supra that the expressed human γ1 results from authentic class switching by genomic recombination between the transgene μ and γ1 switch regions. Furthermore, we have found that intra-transgene class switching was accompanied by somatic mutation of the heavy chain variable regions. In addition to human immunoglobulins, we also found mouse γ and λ in the serum. The present of mouse λ protein is expected because the endogenous locus is completely intact. We have shown elsewhere that the mouse γ expression is a consequence of trans-switch recombination of transgene VDJ segments into the endogenous heavy chain locus. This trans-switching phenomenon, which was originally demonstrated for wild-type heavy chain alleles and rearranged VDJ transgenes (Durdik et al. (1989), *Proc. Natl. Acad. Sci. USA* 86:2346–2350; Gerstein et al. (1990), *Cell* 63:537–548), occurs in the mutant JHD background because the downstream heavy chain constant regions and their respective switch elements are still intact.

The serum concentration of human IgMκ in the double transgenic mice was approximately 0.1 mg/ml, with very little deviation between animals or between lines. However, human γ1, mouse γ, and mouse λ levels range from 0.1 to 10 micrograms/ml. The observed variation in γ levels between individual animals may be a consequence of the fact that γ is an inducible constant region. Expression presumably depends on factors such as the health of the animal, exposure to antigens, and possibly MHC type. The mouse λ serum levels are the only parameter that appears to correlate with individual transgenic lines. KCo4 line 4436 mice which have the fewest number of copies of the transgene per integration (approximately 1–2 copies) have the highest endogenous λ levels, while KCo4 line 4437 mice (~10 copies per integration) have the lowest λ levels. This is consistent with a model in which endogenous λ rearranges subsequent to the κ transgene, and in which the serum λ level is not selected for, but is instead a reflection of the relative size of the precursor B cell pool. Transgene loci containing multiple light chain inserts may have the opportunity to undergo more than one V to J recombination event, with an increased probability that one of them will be functional. Thus high copy lines will have a smaller pool of potential λ cells.

Immunizations With Human CD4 and IgE

To test the ability of the transgenic B cells to participate in an immune response, we immunized double transgenic mice with human protein antigens, and measured serum levels of antigen specific immunoglobulins by ELISA. Mice were immunized with 50 μg recombinant sCD4 (cat. #013101, American Bio-Technologies Inc., Cambridge, Mass.) covalently linked to polystyrene beads (cat #08226, Polysciences Inc., Warrington, Pa.) in complete Freund's adjuvant by intraperitoneal injection. Each of the mice are homozygous for disruptions of the endogenous μ and κ loci, and hemizygous for the human heavy chain transgene HC2 line 2500 and human κ light chain transgene KCo4 line 4437.

Methods

Serum samples were diluted into microtiter wells coated with recombinant sCD4. Human antibodies were detected with peroxidase conjugated rabbit α human IgM(fc) (Jackson Immuno Research, West Grove, Pa.) or peroxidase conjugated goat anti-human Igκ (Sigma, St. Louis, Mo.).

FIG. **71**A shows the primary response of transgenic mice immunized with recombinant human soluble CD4. All four of the immunized animals show an antigen-specific human IgM response at one week. The CD4-specific serum antibodies comprise both human μ heavy chain and human κ light chain.

To evaluate the ability of the HC2 transgene to participate in a secondary response, we hyperimmunized the transgenic mice by repeated injection with antigen, and monitored the heavy chain isotype of the induced antibodies. Mice homozygous for the human heavy chain transgene HC2 and human κ light chain transgene KCo4 were immunized with 25 μg of human IgEκ (The Binding Site, Birmingham, UK) in complete Freund's adjuvant on day=0. Thereafter, animals were injected with IgEκ in incomplete Freund's adjuvant at approximately weekly intervals. Serum samples were diluted 1:10, and antigen-specific ELISAs were performed on human IgE, λ Coated Plates

FIG. 71B shows a typical time course of the immune response from these animals: we injected double transgenic mice with human IgE in complete Freund's adjuvant, followed by weekly boosts of IgE in incomplete Freund's adjuvant. The initial human antibody response was IgMκ, followed by the appearance of antigen specific human IgGκ. The induced serum antibodies in these mice showed no cross-reactivity to human IgM or BSA. The development, over time, of a human IgG □.

We have also tested the ability of the heavy chain transgene to undergo class switching in vitro: splenic B cells purified from animals hemizygous for the same heavy chain construct (HC2, line 2550) switch from human IgM to human IgG1in the presence of LPS and recombinant mouse IL-4. However, in vitro switching did not take place in the presence of LPS and recombinant mouse IL-2, or LPS alone.

We find human IgM-expressing cells in the spleen, lymph nodes, peritoneum, and bone marrow of the double-transgenic/double-knockout (0011) mice. Although the peritoneal cavity contains the normal number of B cells, the absolute number of transgenic B cells in the bone marrow and spleen is approximately 10–50% of normal. The reduction may result from a retardation in transgene-dependent B cell development. The double-transgenic/double-knockout (0011) mice also express fully human antibodies in the serum, with significant levels of human μ, γ1, and κ in these mice. The expressed human γ1 results from authentic class switching by genomic recombination between the transgene μ and γ1 switch regions. Furthermore, the intstransgene class switching is accompanied by somatic mutation of the heavy chain variable regions encoded by the transgene. In addition to human immunoglobulins, we find mouse μ and mouse λ in these mice. The mouse μ expression appears to be a result of trans-switching recombination, wherein transgene VDJ gene is recombined into the endogenous mouse heavy chain locus. Trans-switching, which was originally observed in the literature for wild-type heavy chain alleles and rearranged VDJ transgenes, occurs in our $J_H^{-/-}$ background because the mouse downstream heavy chain constant regions and their respective switch elements are still intact.

To demonstrate the ability of the transgenic B cells to participate in an immune response, we immunized the 0011 mice with human protein antigens, and monitored serum levels of antigen-specific immunoglobulins. The initial human antibody response is IgM, followed by the expression of antigen-specific human IgG (FIGS. 71B and FIG. 73). The lag before appearance of human IgG antibodies is consistent with an association between class-switching and a secondary response to antigen.

In a transgenic mouse immunized with human CD4, human IgG reactivity to the CD4 antigen was detectable at serum concentrations ranging from 2×10^{-2} to 1.6×10^{-4}.

Identification of Anti-human CD4 Hybridomas

A transgenic mouse homozygous for the human heavy chain transgene HC2 and human κ light chain transgene KCo4 were immunized with 20 μg of recombinant human CD4 in complete Freund's adjuvant on day 0. Thereafter, animals were injected with CD4 in incomplete Freund's adjuvant at approximately weekly intervals. FIG. 73 shows human antibody response to human CD4 in serum of the transgenic mouse. Serum samples were diluted 1:50, and antigen-specific ELISAs were performed on human CD4 coated plates. Each line represents individual sample determinations. Solid circles represent IgM, open squares represent IgG.

We also isolated hybridoma cell lines from one of the mice that responded to human CD4 immunization. Five of the cloned hybridomas secrete human IgGκ (human γ1/human κ) antibodies that bind to recombinant human CD4 and do not crossreact (as measured by ELISA) with a panel of other glycoprotein antigens. The association and dissociation rates of the immunizing human CD4 antigen for the monoclonal antibodies secreted by two of the IgGκ hybridomas, 4E4.2 and 2C5.1, were determined. The experimentally-derived binding constants (K_a) were approximately 9×10^7 M^{-1} and 8×10^7 M^{-1} for antibodies 4E4.2 and 2C5.1, respectively. These K_a values fall within the range of murine IgG anti-human CD4 antibodies that have been used in clinical trials by others (Chen et al. (1993) *Int. Immunol.* 6: 647).

A mouse of line #7494 (0012;HC1-26+;JHD++;JKD++; KC2-1610++) was immunized on days 0, 13, 20, 28, 33, and 47 with human CD4, and produced anti-human CD4 antibodies comprised of human κ and human μ or γ.

By day 28, human μ and human κ were found present in the serum. By day 47, the serum response against human CD4 comprised both human μ and human γ, as well as human κ. On day 50, splenocytes were fused with P3X63-Ag8.653 mouse myeloma cells and cultured. Forty-four out of 700 wells (6.3%) contained human γ and/or κ anti-human CD4 monoclonal antibodies. Three of these wells were confirmed to contain human γ anti-CD4 monoclonal antibodies, but lacked human κ chains (presumably expressing mouse λ). Nine of the primary wells contained fully human IgMκ anti-CD4 monoclonal antibodies, and were selected for further characterization. One such hybridoma expressing fully human IgMκ anti-CD4 monoclonal antibodies was designated 2C11-8.

Primary hybridomas were cloned by limiting dilution and assessed for secretion of human μ and κ monoclonal antibodies reactive against CD4. Five of the nine hybridomas remained positive in the CD4 ELISA. The specificity of these human IgMκ monoclonal antibodies for human CD4 was demonstrated by their lack of reactivity with other antigens including ovalbumin, bovine serum albumin, human serum albumin, keyhole limpet hemacyanin, and carcinoembryonic antigen. To determine whether these monoclonal antibodies could recognize CD4 on the surface of cells (i.e., native CD4), supernatants from these five clones were also tested for reactivity with a CD4+ T cell line, Sup T1. Four of the five human IgMκ monoclonal antibodies reacted with these CD4+ cells. To further confirm the specificity of these IgMκ monoclonal antibodies, freshly isolated human peripheral blood lymphocytes (PBL) were stained with these antibodies. Supernatants from clones derived from four of the five primary hybrids bound only to CD4+ lymphocytes and not to CD8+ lymphocytes (FIG. 72).

FIG. 72 shows reactivity of IgMκ anti-CD4 monoclonal antibody with human PBL. Human PBL were incubated with supernatant from each clone or with an isotype matched negative control monoclonal antibody, followed by either a mouse anti-human CD4 monoclonal antibody conjugated to PE (top row) or a mouse anti-human CD8 Ab conjugated to FITC (bottom row). Any bound human IgMκ was detected with a mouse anti-human μ conjugated to FITC or to PE, respectively. Representative results for one of the clones, 2C11-8 (right side) and for the control IgMκ (left side) are shown. As expected, the negative control IgMκ did not react with T cells and the goat anti-human μ reacted with approximately 10% of PBL, which were presumably human B cells.

Good growth and high levels of IgMκ anti-CD4 monoclonal antibody production are important factors in choosing

a clonal hybridoma cell line for development. Data from one of the hybridomas, 2C11-8, shows that up to 5 pg/cell/d can be produced (FIG. 74). Similar results were seen with a second clone. As is commonly observed, production increases dramatically as cells enter stationary phase growth.

FIG. **74** shows cell growth and human IgMκ anti-CD4 monoclonal antibody secretion in small scale cultures. Replicate cultures were seeded at 2×10^5 cells/ml in a total volume of 2 ml. Every twenty-four hours thereafter for four days, cultures were harvested. Cell growth was determined by counting viable cells and IgMκ production was quantitated by an ELISA for total human μ (top panel). The production per cell per day was calculated by dividing the amount of IgMκ by the cell number (bottom panel).

FIG. **75** shows epitope mapping of a human IgMκ anti-CD4 monoclonal antibody. Competition binding flow cytometric experiments were used to localize the epitope recognized by the IgMκ anti-CD4 monoclonal antibody, 2C11-8. For these studies, the mouse anti-CD4 monoclonal antibodies, Leu3a and RPA-T4, which bind to unique, nonoverlapping epitopes on CD4 were used. PE fluorescence of CD4+ cells preincubated with decreasing concentrations of either RPA-TA or Leu-3a followed by staining with 2C11-8 detected with PE-conjugated goat anti-human IgM. There was concentration-dependent competition for the binding of the human IgMκ anti-CD4 monoclonal antibody 2C11-8 by Leu3a but not by RPA-T4 (FIG. **75**). Thus, the epitope recognized by 2C11-8 was similar to or identical with that recognized by monoclonal antibody Leu3a, but distinct from that recognized by RPA-T4.

In summary, we have produced several hybridoma clones that secrete human IgMκ monoclonal antibodies that specifically react with native human CD4 and can be used to discriminate human PBLs into $CD4^+$ and $CD4^-$ subpopulations. At least one of these antibodies binds at or near the epitope defined by monoclonal antibody Leu3a. Monoclonal antibodies directed to this epitope have been shown to inhibit a mixed leukocyte response (Engleman et al., J. Exp. Med. (1981) 153:193). A chimeric version of monoclonal antibody Leu3a has shown some clinical efficacy in patients with mycosis fungoides (Knox et al. (1991) Blood 77:20).

We have isolated cDNA clones from 3 different hybridoma cell lines (2C11.8, 2C5.1, and 4E4.2), and have determined the partial nucleotide sequence of some of the expressed immunoglobulin genes in each of these cell lines.

For sequence analysis, total RNA was isolated from approximately 5×10^6 hybridoma cells. ssc DNA was synthesized by priming reverse transcription with oligo dT. A portion of this ssc DNA was used in duplicate PCR reactions primed by a pool of oligos with specificities for either (i) heavy chain variable framework regions contained within the HC1 or HC2 transgenes and a single downstream oligo specific for constant human gamma sequence, or (ii) light chain variable framework regions contained within the KC2 or KCo4 transgene and a single downstream oligo specific for constant human kappa sequence. Products from these PCR reactions were digested with appropriate restriction enzymes, gel purified, and independently cloned into pNNO3 vector. DNA was isolated and manual dideoxy and/or automated fluorescent sequencing reactions performed on dsDNA.

The characteristics of the three hybridomas, 2C11.8, 2C5.1, and 4E4.2, are given below in Table 11.

TABLE 13

Human variable region usage in hybridomas

Subclone	Specificity	Isotype	Vh	Dh	Jh	$V_κ$	$J_κ$
2C11.8	rCD4	IgMκ	251	nd.*	nd.	nd.	nd.
2C5.1	rCD4	IgGκ	251	HQ52	JH5	65.15	JK4
4E4.2	rCD4	IgGκ	251	HQ52	JH5	65.15	JK4

*n.d., not determined

Nucleotide sequence analysis of expressed heavy and light chain sequences from the two IgGκ hybridomas 2C5.1 and 4E4.2 reveal that they are sibling clones derived from the same progenitor B cell. The heavy and light chain V(DJ) junctions from the two clones are identical, although the precise nucleotide sequences differ by presumptive somatic mutations. The heavy chain VDJ junction sequence is:

```
          VH251                N              DHQ52              JH5
TAT TAC TGT GCG AG    (g gct cc)    A ACT GGG GA     C TGG TTC GAC
  Y  Y  C  A  R         A  P          T  G  D          W  F  D
```

(SEQ ID NOS:176 and 177, respectively)

The light chain VJ junction is:

```
          Vk65.15              N          Jk4
TAT AAT AGT TAC CCT CC       (t)     ACT TTC GGC
  Y  N  S  Y  P  P                    T  F  G
```

(SEQ ID NOS:178 and 179, respectively)

The following non-germline encoded codons were identified (presumptive somatic mutations):

2C5.1	heavy chain	AGC->AGG	S28R	(replacement)
	light chain	CCG->ACG	P119T	(replacement)
4E4.2	heavy chain	AGC->AGG	S28R	(replacement)
		CTG->CTA	L80L	(silent)
	light chain	GAG->GAC	E61D	(replacement)
		AGG->AAG	R61K	(replacement)
		CCG->ACG	P119T	(replacement)

We conclude that these two gamma hybridomas are derived from B cells that have undergone a limited amount of somatic mutation. This data shows that the HC2 transgenic animals use the VH5-51 (aka VH1251) V segment. We have previously shown that VH4-34, VH1-69, and VH3-30.3 are expressed by these mice. The combination of these results demonstrates that the HC2 transgenic @ce expresses all four of the transgene encoded human V_H genes.

We conclude that human immunoglobulin-expressing B cells undergo development and respond to antigen in the context of a mouse immune system. Antigen responsivity leads to immunoglobulin heavy chain isotype switching and variable region somatic mutation. We have also demonstrated that conventional hybridoma technology can be used to obtain monoclonal human sequence antibodies from these mice. Therefore, these transgenic mice represent a source of human antibodies against human target antigens.

Example 37

This example describes the generation of transgenic mice homozygous for an inactivated endogenous heavy chain and K chain locus and harboring a transgene capable of isotype switching to multiple downstream human C_H genes. The example also demonstrates a cloning strategy for assembling large transgenes (e.g., 160 kb) by co-microinjection of multiple DNA fragments comprising overlapping homologous sequence joints (see FIG. **76**), permitting construction of a large transgene from more than two overlapping fragments by homologous recombination of a plurality of homology regions at distal ends of the set of fragments to be assembled in vivo, such as in a microinjected ES cell or its clonal progeny. The example also shows, among other things, that isolated lymphocytes from the transgenic animals can be induced to undergo isotype switching in vitro, such as with IL-4 and LPS.

A set of five different plasmid clones was constructed such that the plasmid inserts could be isolated, substantially free of vector sequences; and such that the inserts together form a single imbricate set of overlapping sequence spanning approximately 150 kb in length. This set includes human V_H D, J, μ, γ3, and γ1 coding sequences, as well as a mouse heavy chain 3' enhancer sequence. The five clones are, in 5' to 3' order: pH3V4D, pCOR1κa, p11-14, pP1-570, and pHP-3a (FIG. **76**). Several different cloning vectors were used to generate this set of clones. Some of the vectors were designed specifically for the purpose of building large transgenes. These vectors (pGP1a, pGP1b, pGP1c, pGP1d, pGP1f, pGP2a, and pGP2b) are pBR322-based plasmids that are maintained at a lower copy number per cell than the pUC vectors (Yanisch-Perron et al. (1985) *Gene* 33: 103–119). The vectors also include trpA transcription termination signals between the polylinker and the 3' end of the plasmid β-lactamase gene. The polylinkers are flanked by restriction sites for the rare-cutting enzyme NotI; thus allowing for the isolation of the insert away from vector sequences prior to embryo microinjection. Inside of the NotI sites, the polylinkers include unique XhoI and SalI sites at either end. The pGP1 vectors are described in Taylor et al. (1992) *Nucleic Acids Res.* 23: 6287. To generate the pGP2 vectors, pGP1f was first digested with AlwNI and ligated with the synthetic oligonucleotides o-236 and o-237 (o-236, 5'-ggc gcg cct tgg cct aag agg cca-3' (SEQ ID NO:180); o-237, 5'-cct ctt agg cca agg cgc gcc tgg-3' (SEQ ID NO:181)) The resulting plasmid is called pGP2a. Plasmid pGP2a was then digested with KpnI and EcoRI, and ligated with the oligonucleotides o-288 and o-289 (o-288, 5'-aat tca gta tcg atg tgg tac-3' (SEQ ID NO:182); o-289, 5'-cac atc gat act g-3' (SEQ ID NO:183)) to crente pGP2b (FIG. **77**A and FIG. **77**B).

The general scheme for transgene construction with the pGP plasmids is outlined in FIG. **78** (paths A and B). All of the component DNA fragments are first cloned individually in the same 5' to 3' orientation in pGP vectors. Insert NotI, XhoI and SalI sites are destroyed by oligonucleotide mutagenesis or if possible by partial digestion, polymerase fill-in, and blunt end ligation. This leaves only the polylinker derived XhoI and SalI sites at the 5' and 3' ends of each insert. Individual inserts can then be combined stepwise by the process of isolating XhoI/SalI fragments from one clone and inserting the isolated fragment into either the 5' XhoI or 3' SalI site of another clone (FIG. **78**, path A). Transformants are then screened by filter hybridization with one or more insert fragments to obtain the assembled clone. Because XhoI/SalI joints cannot be cleaved with either enzyme, the resulting product maintains unique 5' XhoI and 3' SalI sites, and can be used in the step of the construction. A variation of this scheme is carried out using the vectors pGP2a and pGP2b (FIG. **78**, path B). These plasmids includes an SfiI site between the ampicillin resistance gene and the plasmid origin of replication. By cutting with SfiI and XhoI or SalI, inserts can be isolated together with either the drug resistance sequence or the origin of replication. One SfiI/XhoI fragment is ligated to one SfiI/SalI fragment in each step of the synthesis. There are three advantages to this schemne: (i) background transformants are reduced because sequences from both fragments are required for plasmid replication in the presence of ampicillin; (ii) the ligation can only occur in a single 5' to 3' orientation; and (iii) the SfiI ends are not self-compatible, and are not compatible with SalI or XhoI, tbus reducing the level of non-productive ligation. The disadvntage of this scheme is that insert SfiI sites must be removed as well as NotI, XhoI, and SalI sites. These medium copy vectors are an improvement over the commonly used pUC derived cloning vectors. To compare the ability of these vectors to maintain large DNA inserts, a 43 kb XhoI fragment comprising the human JH/Cμ region was ligated into the SalI site of pSP72 (Promega, Madison, Wis.), pUC19 (BRL, Grand Island, N.Y.), and pGP1f. Transformant colonies were transferred to nitrocellulose and insert containing clones were selected by hybridization with radiolabeled probe. Positive clones were grown overnight in 3 ml media and DNA isolated: EcoRI digestion of the resulting DNA reveals that all the pSP72 and pUC19 derived clones deleted the insert (FIG. **79**); however, 12 of the 18 pGP1f derived clones contained intact inserts. Both orientations are represented in these 12 clones.

The construction and isolation of the five clones (pH3V4D, pCOR1κa, p11-14, pP1-570, and pHP-3a) used to generate the HCo7 transgene is outlined below.

pH3V4D.

Germline configuration heavy chain variable gene segments were isolated from phage 1 genomic DNA libraries using synthetic oligonucleotide probes for VH1 and VH3 classes. The VH1 class probe was o-49:

5'-gtt aaa gag gat ttt att cac cec tgt gtc ctc tcc aca ggt gtc-3' (SEQ ID NO:78)

The VH3 class probe was o-184:

5'-gtt tgc agg tgt cca gtg t(c,g)a ggt gca gct g(g,t)t gga gtc (t,c)(g,c)g-3' (SEQ ID NO:184)

Positively hybridizing clones were isolated, partially restriction mapped, subcloned and partially sequenced. From the nucleotide sequence it was determined that one of the VH1 clones isolated with the o-49 probe encoded a VH gene segment, 49.8, comprising an amino acid sequence identical to that contained in the published sequence of the hv1263 gene (Chen et al. (1989) *Arthritis Rheum.* 32: 72). Three of the VH3 genes, 184.3, 184.14, and 184.17, that were isolated with the o-184 probe contained sequences encoding identical amino acid sequences to those contained in the published for the VH genes DP-50, DP-54, and DP-45 (Tomlinson et al. (1992) *J. Mol. Biol.* 227: 776). These four VH genes were used to build the pH3V4D plasmid.

The 184.3 gene was found to be contained within a 3 kb BamHI fragment. This fragment was subcloned into the plasmid vector pGP1f such that the XbaI site of the polylinker is 5' of the gene, and the SalI site is 3'. The resulting plasmid is called p184.3.36f. The 184.14 gene was found to be contained within a 4.8 kb HindIII fragment. This fragment was subcloned into the plasmid vector pUC19 in an orientation such that the gene could be further isolated as a 3.5 kb fragment by XhoI/SalI digestion at a genomic XhoI

described above was digested with restriction enzymes and separated on an agarose gel. Clone pH3V4D was cut with NotI; pCOR1xa was cut with NotI; p11-14 was cut with NotI; pP1-570 was cut with NotI and SalI; and pHP-3a was cut with NotI and XhoI. The DNA inserts were electroeluted and further purified on an equilibrium CsCl gradient without EtBr. The inserts were dialyzed into injection buffer and mixed as follows: 50 microliters of pH3V4D insert @20.4 ng/microliter; 50 microliters of pCOR1xa insert @20.8 ng/microliter; 50 microliters of p11-14 insert @15.6 ng/microliter; 300 microliters of pP1-570 insert @8.8 ng/microliter; 60 microliters of pHP-3a insert @10.8 ng/microliter; and 1.49 ml injection buffer.

HCo7 Transgenic Animals

The HCo7 DNA mixture was microinjected into the pronuclei of one-half day old embryos, and the embryos transferred into the oviducts of pseudopregnant females, as described by Hogan et al. (Manipulating the mouse embryo, Cold Spring Harbor laboratories, Cold Spring Harbor N.Y.).

Tail tip DNA was isolated from 202 animals that developed from microinjected embryos. Southern blot analysis of this DNA, using a probe comprising human μ and DH sequences, revealed 22 founder animals that had incorporated at least a portion of the HCo7 transgene. FIG. **81** shows an analysis of the expression of human μ and human γ1 in the serum of 6 GO animals that developed from embryos microinjected with HCo7 DNA. Serum levels of human immunoglobulin proteins were measured by ELISA as described in Lonberg et al. (1994) *Nature* 368: 856. Four of these six mice showed evidence of incorporation of the transgene by Southern blot analysis, and three of these mice expressed both human μ and human γ1 proteins in their serum. The single transgenic mouse that did not express human immunoglobulin proteins was determined by Southern blot analysis to contain only a low number of copies of the transgene, and it is possible that the entire transgene was not incorporated, or that this mouse was a genetic mosaic. Two of the founder HCo7 mice, #11952 and #11959, were bred with human κ minilocus (KCo4 line 4436) transgenic mice that were also homozygous for disruptions of the endogenous heavy, and K light chain loci (Lonberg et al. op.cit), to generate mice that were homozygous for the two endogenous locus disruptions and hemizygous for the two introduced human miniloci, KCo4 and HCo7. Five of these so-called double-transgenic/double-deletion mice were analyzed for expression of human IgM, human IgG1, and human IgG3. As a control, three HC2/KCo4 double-transgenic/double-deletion mice were included in the analysis. This experiment is presented in FIG. **82**. The ELISA data in this figure was collected as in Lonberg et al. (op.cit), except that for detection of human IgG3, the coating antibody was a specific mAb directed against human IgG3 (cat. #08041, Pharmingen, La Jolla, Calif.); the other details of the IgG3 assay were identical to those published for IgG1. While the HC2/KCo4 mice express only human IgM and human IgG1, the HCo7/KCo4 mice also express human IgG3 in addition to these two isotypes. Expression of human γ3 and γ1 in the HCo7 mice has also been detected by PCR amplification of cDNA synthesized from RNA isolated from the spleen of a transgenic mouse. FIG. **83** depicts PCR amplification products synthesized using spleen cDNA from three different lines of transgenic mice: line 2550 is an HC2 transgenic line, while lines 11959 and 11952 are HCo7 transgenic lines. Single stranded cDNA was synthesized from spleen RNA as described by Taylor et al. (1992) *Nucleic Acid Res.* 20: 6287. The cDNA was then PCR amplified using the following two oligonucleotides:

o-382: 5'-gtc cag aat tcg gt(c,g,t) cag ctg gtg (c,g)ag tct gg-3' (SEQ ID NO:190)

o-383: 5'-ggt ttc tcg agg agg agg agg aag act gac ggt cc-3' (SEQ ID NO:190)

This primer pair directs the synthesis of PCR products that spans the hinge region of human γ transcripts. Because of differences in the structures of the human γ1 and γ3 hinge regions, PCR amplification distinguishes between these two transcripts. A human γ1 template will direct the synthesis of a 752 bp PCR product, while human γ3 directs the synthesis of a 893 bp product. While only human γ1 template is detectable in the HC2 line 2550 and HCo7 line 11959 spleens, both γ1 and γ3 transcripts are detectable in the HCo7 line 11952 spleen. Because of the non-quantitative nature of this assay, and because of differences in γ3 expression between individual animals (shown by ELISA in FIG. **82**), the inability to observe γ3 in the HCo7 line 11959 spleen in FIG. **83** does not indicate that γ3 is not expressed in this line. Isolated spleen cells from the HCo7/KCo4 mice can also be induced to express both IgG1 and IgG3 in vitro by stimulation with LPS and IL4. This experiment is shown in FIG. **84**. Spleen cells from a 7 week old male HCo7/KCo4 double-transgenic/double-deletion mouse (#12496; line 11959/4436) tested for immunoglobulin secretion in response to the thymus-independent B cell mitogen, LPS, alone and in conjunction with various cytokines. Splenocytes were enriched for B cells by cytotoxic elimination of T cells. B-enriched cells were plated in 24 well plates at 2×10^6 cells per well in 2 ml of 10% FCS in RPMI-1640. LPS was added to all wells at 10 micrograms/ml. IL-2 was added at 50 units/ml, IL-4 was added at 15 ng/ml, IL-6 was added at 15 ng/ml, γIFN was added at 100 units/ml. Cultures were incubated at 37° C., 5% CO_2 for 10 days, then supernatants were analyzed for human IgG1 and IgG3 by ELISA. All reagents for ELISA were polyclonal anti-serum from Jackson Immunologicals (West Grove, Pa.), except the capture anti-human IgM, which was a monoclonal antibody from The Binding Site (Birmingham, UK).

Example 38

This example demonstrates the successful introduction into the mouse genome of functional human light chain V segments by co-injection of a human κ light chain minilocus and a YAC clone comprising multiple human V_κ segments. The example shows that the V_κ segment genes contained on the YAC contribute to the expressed repertoire of human κ chains in the resultant mouse. The example demonstrates a method for repertoire expansion of transgene-encoded human immunoglobulin proteins, and specifically shows how a human κ chain variable region repertoire can be expanded by co-introduction of unlinked polynucleotides comprising human immunoglobulin variable region segments.

Introduction of Functional Human Light Chain V Segments by Co-injection of Vk Containing Yeast Artificial Chromosome Clone DNA and k Light Chain Minilocus Clone DNA

I. Analysis of a Yeast Strain Containing Cloned Human Vk Gene Segments.

Total genomic DNA was isolated from a yeast strain containing a 450 kb yeast artificial chromosome (YAC) comprising a portion of the human V_κ locus (ICRF YAC library designation 4x17E1). To determine the identity of some of the V_κ gene segments included in this YAC clone,

mouse #12648 (HC2-2550/KCo5-9272/JHD/JKD) was sacrificed and total RNA isolated from the spleen. Single stranded cDNA was synthesized from the RNA and used as a template in four separate PCR reactions using oligonucleotides o-270, o-271, o-272, and o-273 as 5' primers, and the Ck specific oligonucleotide, o-186 (5'-tag aag gaa ttc agc agg cac aca aca gag gca gtt cca-3'; SEQ ID NO:173), as a 3' primer. The amplification products were cloned into the pCR11 TA cloning vector (Invitrogen). The nucleotide sequence of 19 inserts was determined. The results of the sequence analysis are summarized in Table 15 below.

TABLE 15

Identification of human Vk genes expressed in mouse line KCo5-9272.

PCR primers	clone #	identified gene	Vk family
o-270/o-186	1	L15*	I
"	3	L18**	I
"	7	L24**	I
"	9	L15*	I
"	10	L15*	I
o-271/o-186	15	A10**	VI
"	17	A10**	VI
"	18	A10**	VI
"	19	A10**	VI
"	21	A10**	VI
o-272/o-186	101	A27*	III
"	102	L15*	I
"	103	A27*	III
"	104	A27*	III
o-273/o-186	35	A27*	III
"	38	A27*	III
"	44	A27*	III
"	45	A27*	III
"	48	A27*	III

*Vk genes encoded by transgene plasmid sequences.
**Vk genes encoded uniquely by YAC derived transgene sequences.

These results show that at least 3 of the YAC derived V_k gene segments, A10, L18, and L24, contribute to the expressed human repertoire of the line KCo5-9272 mice.

To determine the effect of this increased repertoire on the size of the various B220$^+$ cell populations in the bone marrow and spleen, a flow cytometric analysis was carried out on line KCo5-9272 mice. Part of this analysis is shown in FIGS. **86** and **87**. Two double transgenic/double deletion mice, one containing the KCo5 transgene, and one containing the KCo4 transgene, are compared in this experiment. These two transgenes share the same joining and constant region sequences, as well as the same intronic and 3' enhancer sequences. They also share four different cloned V gene segments; however, the KCo5 transgene includes the additional V segments derived from YAC 4x17E1 that are not included in the KCo4 transgene. Cells were isolated from mouse #13534 (HC2-2550/KCo5-9272/JHD/JKD) and mouse #13449 (HC2-2550/KCo4-4436/JHD/JKD). Bone marrow cells were stained with anti-mouse B220 (Caltag, South San Francisco, Calif.), anti-mouse CD43 (Pharmingen, La Jolla, Calif.), and anti-human IgM (Jackson Immunologic, West Grove, Pa.). Spleen cells were stained with anti-mouse B220 and anti-human IgM.

FIG. **86** shows a comparison of the B cell, and B cell progenitor populations in the bone marrow of KCo5 and KCo4 mice. The fraction of B cells in the bone marrow (B220$^+$, IgM$^+$) is approximately three times higher in the KCo5 mice (9%) than it is in the KCo4 mice (2%). The pre-B cell population (B220$^+$, CD43$^-$, IgM$^-$) is also higher in the KCo5 mice (9%, compared to 5% for KCo4). Furthermore, the pro-B compartment (B220$^+$, CD43$^+$) is elevated in these mice (11% for KCo5 and 5% for KCo4). Although each of these three compartments is larger in the KCo5 mice than it is in the KCo4 mice, the levels are still approximately half that found in wild type mice. The increase in the number of bone marrow B cells is presumably a direct consequence of the increased repertoire size. The larger primary repertoire of these mice may provide for membrane Ig with some minimal threshold affinity for endogenous antigens. Receptor ligation could then allow for proliferation of those B cells expressing the reactive Ig. However, because the pro-B and pro-B cells do not express light chain genes, the explanation for the increased sizes of these two compartments in the KCo5 mice is not immediately apparent. The B cell progenitor compartments may be larger in KCo5 mice because the increased number of B cells creates a bone marrow environment that is more conducive to the expansion of these populations. This effect could be mediated directly by secreted factors or by cell-cell contact between B cells and progenitor cells, or it could be mediated indirectly, by titration of factors or cells that would otherwise inhibit the survival or proliferation of the progenitor cells.

FIG. **87** shows a comparison of the splenic B cell (B220$^+$, IgM$^+$) populations in KCo5 and KCo4 mice. The major difference between these two mice is the relative sizes of B220^{dull} B cell populations (6% in the KCo5 mice and 13% in the KCo4 mice). The B220^{dull} cells are larger than the B220^{bright} B cells, and a higher fraction of them express the 1 light chain. These are characteristics of the so-called B1 population that normally dominates the peritoneal B cell population in wild type mice. The spleens of the KCo4 mice comprise an anomalously high fraction of B220^{dull} cells, while the KCo5 mice have a more normal distribution these cells. However, both strains contain approximately one-half to one-third the normal number of B cells in the spleen.

Example 39

This example demonstrates the successful use of KCo5 transgenic mice of Example 38 to isolate hybridoma clones that secrete high affinity, antigen specific, human IgG monoclonal antibodies.

Immunization

A double deletion/double transgenic mouse (KCo5-9272/HC2-2550/JHD/JKD), #12657) was immunized intraperitoneally every other week for eight weeks with 4 to $10x10^6$ irradiated T4D3 cells, a murine T cell line expressing human CD4 (Dr. Jane Parnes, Stanford University) followed by one injection intraperitoneally two weeks later of 20 mg soluble recombinant human CD4 (sCD4; Intracell) in incomplete Freund's adjuvant (Sigma). The mouse was boosted once 3 days prior to fusion with 20 mg sCD4 intravenously.

Hybridoma Fusion

Single cell suspensions of splenic lymphocytes from the immunized mouse were fused to one-sixth the number of P3X63-Ag8.653 nonsecreting mouse myeloma cells (ATCC CRL 1580) with 50% PEG (Sigma). Cells were plated at approximately $2x10^5$ in flat bottom microtiter plates, followed by a two week incubation in selective medium containing 20% Fetal Clone Serum (HyClone), 18% "653" conditioned medium, 5% Origen (IGEN), 4 mM L-glutamine, 1 mM sodium pyruvate, 5 mM HEPES, 0.055 mM 2-mercaptoethanol, 50 units/ml mM penicillin, 50 mg/ml streptomycin, 50 mg/ml mM gentamycin and 1xHAT (Sigma; the HAT was added 24 hrs after the fusion). After

two weeks, cells were cultured in medium in which the HAT was replaced with HT. Wells were screened by ELISA and flow cytometry once extensive hybridoma growth or spent medium was observed.

Hybridoma Screening by ELISA

To detect anti-CD4 mAbs, microtiter plates (Falcon) were coated overnight at 4° C. with 50 ml of 2.5 mg/ml of sCD4 in PBS, blocked at RT for 1 hr with 100 ml of 5% chicken serum in PBS, and then sequentially incubated at RT for 1 hr each with 1:4 dilutions of supernatant from hybridomas, 1:1000 dilution of $F(ab')_2$ fragments of horseradish peroxidase (HRPO)-conjugated goat anti-human IgG (Jackson) or 1:250 dilution of HRPO-conjugated goat anti-human Igk antibodies (Sigma) plus 1% normal mouse serum, and finally with 0.22 mg/ml ABTS in 0.1 M citrate phosphate buffer, pH 4 with 0.0024% H_2O_2. Plates were washed 3–6 times with wash buffer (0.5% Tween-20 in PBS) between all incubations, except the first. Diluent (wash buffer with 5% chicken serum) was used to dilute the supernatants and the HRPO conjugates. Absorbance was measured using dual wavelengths (OD at the reference wavelength of 490 nm was subtracted from the OD at 415 nm).

To detect mouse λ-containing mAbs, the above ELISA protocol was used, with the following exceptions.

Wells of microtiter plates were coated with 100 ml of 1) 1.25 mg/ml goat anti-mouse λ (Pierce), 2) 1.25 mg/ml goat anti-human Fcγ (Jackson), or 3) 2.5 mg/ml sCD4 (ABT).

For the detection step, 100 ml of 1:5000 goat anti-mouse l (SBA) conjugated to biotin was used followed by 100 ml of 1:1000 streptavidin conjugated to HRPO (Jackson).

Murine and human mAb standards were used at the indicated concentrations. To look for cross-reactivity to unrelated antigens, wells were coated with CEA (Crystal Chem), KLH (CalBiochem), HS_4 (Sigma), BSA (Sigma) or OVA (Sigma; all at 2 mg/ml, except CEA which was at 2.5).

Appropriate antibodies were titered and used as positive controls (human IgM anti-CEA (GenPharm), rabbit anti-KLH (Sigma), sheep anti-HSA (The Binding Site), sheep anti-BSA (The Binding Site), and sheep anti-OVA (The Binding Site)). Any bound antibody was detected with HRPO conjugates of goat anti-human IgM, donkey anti-rabbit IgG or donkey anti-sheep IgG (all diluted 1:1000 and obtained from Jackson). Otherwise, the standard ELISA protocol was followed.

Hybridoma Screening by Flow Cytometric Assay

To further screen for mAbs reactive with native cell-surface CD4, $5x10^5$ SupT1 cells (ATCC CRL 1942) were incubated on ice with a 1:2 dilution of spent supernatant from the fusion plates for 30 min, washed twice with cold stain buffer (0.1% BSA, 0.02% NaN_3 in PBS), incubated with 1.5 mg/ml of an $F(ab')_2$ fragment of FITC-conjugated goat anti-human Fcg (FITC-GaHulgG; Jackson) for 15 min, washed once and analyzed immediately on a FACScan (Becton-Dickinson).

CD4 Reactive Hybridomas

Using the ELISA and flow cytometric techniques described above, 12 hybridoma clones were identified that secreted human IgG specifically reactive with native human CD4. Ten of these twelve clones were further subcloned. Eight of these subclones were identified as human IgG1 κ secreting hybridomas. The other two expressed a mouse λ light chain. The parent wells for the 8 fully human clones were: 1E11, 2F4, 4D1, 6C1, 6G5, 7G2, 10C5, and 1G1. Flow cytometric assays of the binding of 3 of the fully human IgGk subclones (4D1.4, 6G5.1, and 10C5.6) are shown in FIG. **88**.

FIG. **88** shows binding of IgGκ anti-nCD4 monoclonal antibodies to CD4+ SupT1 cells. Cells from log phase growth cultures were washed and stained with no monoclonal antibody, 4E4.2 (as a negative control), chimeric Leu3a (as a positive control), or with one of the 10 human IgG anti-nCD4 monoclonal antibodies. Any bound monoclonal antibody was detected with FITC-conjugated goat anti-human Fcγ. All ten monoclonal antibodies bound to SupT1 cells, although data is shown here for only three of them.

Analysis of Human Antibody Secretion by Cloned Hybridomas

To compare the growth and secretion levels of mAbs, the subclones were put into replicate cultures in HT medium in 24 well plates at an initial density of $2x10^5$ cells/ml. Each day for 7 days, one of the replicate cultures for each subclone was harvested and cell numbers, cell viability (by Trypan blue exclusion) and the amount of mAb in the supernatant (by a quantitative ELISA for total human γ) were determined. Table 16 shows data for antibody secretion by 7 of the hybridoma subclones.

TABLE 16

Secretion Levels For Human IgGk Anti-nCD4 Monoclonal Antibodies

Subclone	pg/cell	pg/cell/d
1E11.15	3.9	0.56
1G1.9	11	1.5
4D1.4	1.4	0.91
6C1.10	3.3	0.48
6G5.1	7.8	1.1
7G2.2	4.4	0.63
10C5.6	8.0	1.1

* pg/cell = (maximum amount of mAb)/(maximum number of viable cells)
pg/cell/d = (pg/cell)/7 days

Purification of Human mAbs

The individual hybridoma clones were grown in medium without HT and Origen and the FCS was gradually decreased to approximately 2–3% in the final 1 l cultures. Supernatants were harvested once the viability of the hybridomas fell below approximately 30%. To purify the IgGk mAbs, the spent supernatants were centrifuged to remove cells, concentrated via ultrafiltration to approximately 50 to 100 mls, diluted 1:5 with PBS, pH 7.4 and loaded onto a 5 ml Protein A (Pharmacia) column. After washing with 3–5 column volumes of PBS, the human IgGk mAbs were eluted with 0.1 HCl, 150 mM NaCl, pH 2.8 and immediately neutralized with 1M Tris base. Column fractions containing material with an OD_{280}>0.2 were pooled and dialyzed into PBS. The OD_{280} was then determined and an absorbivity coefficient of 1.4 was used to calculate the protein concentration of the human IgG. No mAb was detected in the flow through and the % recoveries ranged from 93 to 100%. Three to six mgs of each purified mAb were obtained, with >90% purity.

Analysis of Monoclonal Antibodies From Cloned Hybridomas

To investigate the specificity of binding of mAbs, human PBMC were isolated over Ficoll and stained as follows.

Human PBMC (10^6) in stain buffer were incubated for 30 min on ice, in scparate reactions, with equal volumes of supernatant from each of three of the subcloned hybridomas (4D1.4, 6G5.1, and 10C5.6), or with an isotype matched negative control mAb, washed twice, and incubated 20 min on ice with 1 mg/ml of FITC-GaHulgG along with either 10 ml of mouse anti-human CD4 mAb (Leu3a; Becton-Dickinson) conjugated to phycoerythrin (PE), 10 ml of mouse anti-human CD8 mAb (Leu2a; Becton-Dickinson) conjugated to PE, or 5 ml of mouse anti-human CD19 mAb (SJ25-C1; Caltag) conjugated to PE. Gated lymphocytes were then analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). All three of the antibodies were found to bind specifically to the CD4 fraction of the human PBMC.

To approximate the location of the epitope recognized by these three mAbs, 5×10^5 SupT1 cells were pre-incubated for 20 min on ice with buffer, 2.5 mg/ml RPA-T4, or 2.5 mg/ml Leu3a in stain buffer, then for 30 min with one of the 10 human IgG mAbs (in supernatant diluted 1:2) and finally with 0.5 mg/ml FITC-conjugated goat anti-human Fcγ to detect any bound human IgG. Cells were washed twice with stain buffer prior to and once after the last step. The results of this blocking assay are shown in FIG. **89**. None of the three antibodies share an epitope with RPA-T4, while 6G5.1 and 10C5.6 appear to recognize the same (or an adjacent) epitope as that recognized by Leu3a.

Rate and Equilibrium Constant Determinations

Human sCD4 (2500 to 4200 RU) was immobilized by covalent coupling through amine groups to the sensor chip surface according to manufacturer's instructions. Antibody dilutions were flowed over the antigen-coupled sensor chips until equilibrium was reached, and then buffer only was allowed to flow. For each phase of the reaction, binding and dissociation, the fraction of bound antibody was plotted over time. The derivative of the binding curve (dR/dt) was calculated and plotted against the response for each concentration. To calculate the association rate constant (k_{assoc}), the slopes of those resulting lines were then plotted against the concentration of the monoclonal antibody. The slope of the line from this graph corresponded to the k_{assoc}. The dissociation rate constant (k_{dissoc}) was calculated from the log of the drop in response (during the buffer flow phase) against the time interval. The Ka was derived by dividing the k_{assoc} by the k_{dissoc}. The measured rate and affinity constant data for 5 different purified monoclonal antibodies derived from the KCo5/HC2 double transgenic/double deletion mice, and one purified antibody obtained from a commercial source (Becton Dickinson, San Jose, Calif.), is presented in Table 17.

TABLE 17

Rate and affinity constants for monoclonal antibodies that bind to human CD4.

Hybridoma	Antibody	Source	k_{ass} ($M^{-1}s^{-1}$)	k_{dis} (s^{-1})	Ka (M^{-1})
1E11.15	human IgG1k	HC2/KCo5 transgenic	2.7×10^5	4.6×10^{-5}	5.8×10^9
1G1.9	human IgG1k	HC2/KCo5 transgenic	9.1×10^4	2.2×10^{-5}	4.2×10^9
4D1.4	human IgG1k	HC2/KCo5 transgenic	9.8×10^4	4.2×10^{-5}	2.3×10^9
6G5.1	human IgG1k	HC2/KCo5 transgenic	1.1×10^5	1.0×10^{-5}	1.1×10^{10}

TABLE 17-continued

Rate and affinity constants for monoclonal antibodies that bind to human CD4.

Hybridoma	Antibody	Source	k_{ass} ($M^{-1}s^{-1}$)	k_{dis} (s^{-1})	Ka (M^{-1})
10C5.6	human IgG1k	HC2/KCo5 transgenic	7.4×10^4	1.6×10^{-5}	4.5×10^9
Leu3a	mouse IgG1k	Becton Dickinson	1.5×10^5	4.2×10^{-6}	3.7×10^{10}

Mixed Lymphocyte Reaction (MLR)

To compare the in vitro efficacy of the human monoclonal antibody 10C5.6, derived from the KCo5 transgenic mouse, to that of the mouse antibody Leu3a, an MLR assay was performed. Human PBMC from 2 unrelated donors were isolated over Ficoll and CD4+ PBL from each donor were purified using a CD4 column (Human CD4 Cellect, Biotex Laboratories, Inc., Canada) according to manufacturer's directions. Inactivated stimulator cells were obtained by treating PBMC from both donors with 100 mg/ml mitomycin C (Aldrich) in culture medium (RPMI 1640 with 10% heat-inactivated human AB serum (from NABI), Hepes, sodium pyruvate, glutamine, penstrep and b-mercaptoethanol (all used at manufacturer's recommended concentrations)) for 30 min at 37° C. followed by 3 washes with culture medium. Varying concentrations of mAbs diluted in culture medium or culture medium only were sterile filtered and added at 100 ml per well in triplicate in a 96 well round bottom plate. Fifty ml of 10^5 CD4+ PBL from one donor in culture medium and 10^5 mitomycin C-treated PBMC from the other donor in 50 ml of culture medium were then added to each well. Control plates with CD4+ PBL responders alone plus mAbs were set up to control for any toxic or mitogenic effects of the mAbs. A stimulator only control and a media background control were also included. After seven days in a 37° C., 5% CO_2 humidified incubator, 100 ml of supernatant from each well was removed and 20 ml of Calorimetric reagent (Cell Titer 96AQ kit, Promega Corporation, Madison, Wis.) was added. Color was allowed to develop for 4 to 6 hrs and plates were read at 490 nm. The results of this experiment, depicted in FIG. **90**, show that the human IgG1 k antibody 10C5.6 is at least as effective as Leu3a at blocking the function of human PBMC CD4 cells in this assay.

Example 40

Binding Characteristics of Human IgGkappa Anti-CD4 Monoclonal Antibodies

This example provides the binding characteristics of human IgGκ monoclonal antibodies derived from hybridoma clones obtained from HC2/KCo5/JHD/JCKD transgenic mice immunized with human CD4. The monoclonal antibodies are shown to have high avidity and affinity for recombinant and natural human CD4.

Cells from 10 individual hybridoma cell lines (1E11, 1G2, 6G5, 10C5, 1G1, 6C1, 2E4, 7G2, 1F8 and 4D1) that secrete human IgG kappa monoclonal antibodies (mAb) reactive with human CD4, were derived from JHD/JCKD/HC2/KCo5 transgenic mice. The cell lines were grown in culture, and antibody proteins were isolated from the supernatant (Fishwild, et al. 1996, *Nature Biotechnology* 14, 845–851, which is incorporated herein by reference). Antibody puri-